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The development of intestinal microbiota in childhood and host-microbe interactions in pediatric celiac disease

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ACADEMIC DISSERTATION

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The publications and corresponding studies are referred in the text by their Roman number. The original articles are reprinted by the permission of the publishers. * These authors contributed equally in the publications.

Abbreviation

AMP	Antimicrobial peptide
BF/ FF	Breast-feeding/ formula-feeding
BMI	Body mass index
CeD	Celiac disease
CXCL16	Chemokine (C-X-C motif) ligand 16
CXCR6	C-X-C motif chemokine receptor 6
EMA	Endomysial antibodies
FUT2	Fucosyltransferase gene 2
GALT	Gut-associated lymphoid tissue
HITChip	Human Intestinal Tract Chip
HLA	Human Leukocyte Antigen
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
LP	Lamina propria
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAMP	Microbe-associated molecular pattern
NLR	NOD-like receptor
NF- kB	Nuclear factor-kB
NOD2	Nucleotide-oligomerization-domain-2
OTU	Operational taxonomic unit
PP	Peyer's patches
PRR	Pattern recognition receptor
RegIII	Regenerating islet-derived protein 3
SCFA	Short-chain fatty acid
sIgA	Secretory immunoglobulin A
SNP	Single nucleotide polymorphism
T1D	Type I diabetes
TJ	Tight junction
TNF	Tumor necrosis factor
Tollip	Toll interacting protein
TLR	Toll-like receptor
Treg	Regulatory T cell
tTG	Tissue transglutaminase
ZO	Zonula-occludens

Abstract

The interactions between a host and his/her microbiota have co-evolved over time and they exert profound effects on each other. Intestinal microbiota has been linked with a number of diseases, such as irritable bowel syndrome; it is considered to be a major etiopathological factor since it can alter intestinal homeostasis. However, the role of intestinal microbiota, especially commensals, is unclear in celiac disease. To date, most efforts for detecting potential microbial changes affected by celiac disease have focused on adult individuals and have examined fecal materials, although it is known that early life is the critical period for the microbiota to colonize and establish their niche in the human intestine. At this time in healthy individuals, there is continuing cross-talk with the host e.g. via the immune system, leading to the establishment of homeostasis in both metabolic and immunological programming. Since the intestinal epithelium is the main interface for host-microbe interactions, the role of mucosa-associated microbiota may be distinct from that of fecal microbiota, but both the normal fluctuations in intestinal microbiota and the composition of duodenal mucosa-associated microbiota are still not fully clarified.

The aims of thesis were to characterize the development and stability of intestinal microbiota in healthy young children and to compare the microbial features between children and adults. Furthermore, the aim was to investigate host-microbe interactions in celiac disease by studying duodenal mucosa-associated microbial signatures and mucosal gene expression in healthy children and their counterparts with celiac disease. The microbiota profiles were characterized by using the human intestinal tract chip (HITChip), which is a bacterial phylogenetic microarray. The amounts of *Bifidobacterium* spp. in children and adults were verified with real time qPCR. The levels of mucosal gene expressions were quantified with reverse transcriptase quantitative PCR.

The results showed that intestinal microbiota is not fully matured at the age of five in children. A common core microbiota, including several butyrate-producing bacteria, was identified in children and it was developing towards core microbiota found in adults. The different progression pattern of major bacterial taxa may reflect the physiological development steps in children. Moreover, differences were observed between healthy- and celiac disease- associated microbial signatures. The differences may reflect changes in epithelial integrity associated with the disease. On the other hand, the studies on both microbiota and mucosal gene expression indicated that the persistently enhanced Th1 type immune responsiveness in subjects with celiac disease after treating with gluten-free diet might result from the increased expression of TLR9, which recognizes unmethylated CpG motifs in bacterial DNA via the direct stimulation of immune cells and/or intestinal epithelial cells.

The results of this thesis project suggest that specific symbiotic and dysbiotic microbial signatures may provide potential functional diagnostic or therapeutic targets for promoting healthy/natural microbiota development. Long-term studies in a controlled environment with an adequate number of participants will be necessary to decode the disturbed microbial signatures. These trials should be combined with systematic pathological surveillance to reveal how the changes in the microbiota influence the onset of disease.

1. INTRODUCTION

In the past 50 years, human life-styles and diet in Western countries have undergone a huge transformation. Urbanization, industrialization and medical practices, such as the use of antibiotics, have led to excessively hygienic living conditions (Campbell 2014; Haahtela et al. 2013). The current hygiene hypothesis states that early life exposure to microorganisms, such as commensals, is crucial for immunological and metabolic programming, and that if this exposure is inadequate or proceeds in an aberrant manner, it can contribute to the appearance of the immunoregulatory defects that underlie the increased prevalence of chronic inflammatory disorders now so common in the developed countries (Isolauri et al. 2009). Examples of disorders linked to the hygienic hypothesis are allergies and autoimmune diseases, such as type 1 diabetes (T1D), Celiac disease (CeD) and inflammatory bowel disease (IBD), particularly Crohn's disease (Campbell 2014; Prescott 2013). Among these diseases, chronic intestinal mucosal inflammation is the main histological and clinical manifestation of CeD and IBD, as either a cause or a consequence of disrupted intestinal (or gut) homeostasis, i.e. changes in the host-microbe interactions. This palette of symptoms refers to dys-regulated immunity, impaired physical barrier function or microbial dysbiosis.

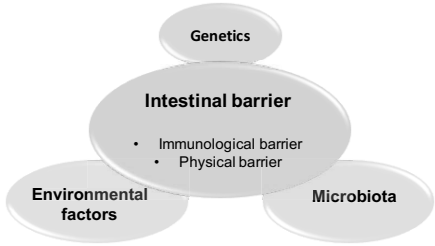

Figure 1. Schematic representation of different factors in health and diseases.

Intestinal barrier, including both physical and immunological barrier, has evolved to prevent invasion of endogenous or exogenous irritants from intestinal lumen to the host, such as microbes and chemical toxicants (Bischoff et al. 2014) (Fig. 1). The co-evolution of gastrointestinal (GI) microbes and their mammalian hosts can influence which microbial species and how many of them are able to colonise within the host and this then modulates the functions of intestinal barriers of the host (Fig. 1). Evolutionary interactions with microbes are considered as key contributors to intestinal homeostasis which is essential for the host to remain healthy (Sommer et al. 2014). These beneficial interactions are dynamic processes during the development of host physiology, initiated immediately when the first microbes colonize the host intestine (Rautava, Luoto, et al. 2012). Although these interactions are active constantly throughout the whole life span, early life in childhood has been postulated to be crucial for the shaping of both the host metabolism and the immune system, introducing profound short and long term effects on host health (Sommer and Bäckhed 2013; Rautava, Luoto, et al. 2012).

Celiac disease (CeD) is an autoimmune disorder, which often starts during childhood and lasts for the individual's whole life. The disease is triggered by dietary gluten and characterized by inflammation of the small intestine. The pathogenesis of the disease is thought to be multifactorial involving the intolerance

of gluten via complex interactions between the host's genetic predisposition, a microbial dysbiosis and environmental factors (Fig. 1). In particular, commensal bacteria may play a crucial role in celiac disease as they can maintain the intestinal barrier and modulate many immunological functions (Cinova et al. 2011; Galipeau et al. 2014; Lindfors et al. 2008).

In order to obtain a better understanding of the complex host-microbe interactions in pediatric celiac disease, it is crucial to characterize the microbiota in childhood and understand whether the disease state can be related to changes in certain microbes. With the development of high-throughput molecular profiling methods, it is now possible to undertake a holistic characterization of intestinal microbiota in both healthy subjects and CeD patients. The aims of thesis were to characterize the development of intestinal microbiota in healthy children and to investigate host-microbe interactions in celiac disease.

2. LITERATURE REVIEW

2.1 Host-microbe interactions

The human GI-tract is composed of the stomach, small intestine and large intestine. Together with other digestive organs, such as liver and pancreas, the human GI-tract makes up the digestive system (Fig. 2).

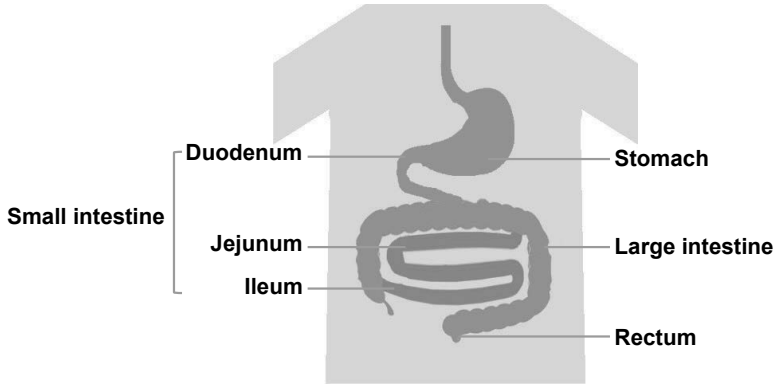

Figure 2. Schematic representation of different regions of GI-tract.

This system is responsible for breaking down food and subsequently absorbing nutrients. The human being and his/her intestinal microbiota, i.e. the populations of microbes found in the gastrointestinal tract, form a so-called "superorganism" or "holobiont", in which both parts mutually strive for survival (Relman 2012). The mucus layer, which coats the entire GI-tract, is the main location for host-microbe interactions. In the small intestine, the enterocytes of the villous epithelium are the main interface for digestive activities. In addition, the mucus layer of the whole GI-tract also serves as a defence barrier to protect the host from harmful antigens, toxins and microbes. These distinct structural, digestive and protective functions constitute heterogeneous microenvironments, which in turn affect the composition of microorganisms attached to the epithelium or those moving along with the food during the digestion (Table 1).

2.1.1 How the host impacts on the intestinal microbiota

The ways that the host exerts effects on the microbiota are multiplex. Within the different sections of an individual's GI-tract, the differences in pH, oxygen level and mucus contents, are major elements affecting bacterial density and complexity (Table 1). Furthermore, long-term changes in the physiology of the whole body, from childhood to old age, are reflected in the structure of intestinal microbiota as has been observed when studying differences between healthy children, adults and elderly subjects (Sommer and Bäckhed 2013; Odamaki et al. 2016). In addition, host genotypes, environment (including diets) and normal GI-tract conditions can all affect the composition of microbial community, which are elaborated later in this section. Finally, host pathology has a profound effect on the microbiota composition, and sometimes could even dominate all of these other factors (see Section 2.1.3).

		Microbiota Characteristics			Host Environment		
		Diversity	Density (cfu/g)	Main Taxa	pH	Mucin	Oxygen
Stomach		Low	10^1-10^3	*Prevotella* *Streptococcus*	<3	MUC5	High
Small intestine	Duodenum		$<10^4$	*Prevotella* *Streptococcus* *Veillonella*	6.4	MUC2	
	Jejunum		10^4-10^5	*Streptococcus* *Veillonella* γ- *Proteobacteria*	6.6	MUC2	
	Ileum		10^7-10^8	*Streptococcus* Bacteroidetes *Clostridium* cluster IV *Clostridium* cluster XIVa	7.3	MUC2	
Large intestine		High	10^{10}-10^{12}	Actinobacteria Bacteroidetes Clostridium	5.7-6.6	MUC2	Low

Table 1. Characteristics of microbiota and host environment along GI tract. The density of microbiota is defined as the number of bacterial cells per gram of intestinal content. The bacterial taxa in the table include both luminal and mucosal taxa (McGuckin et al. 2011; Lozupone et al. 2012; Ohland and Jobin 2015; Zoetendal et al. 2012; Bik et al. 2006; Islam et al. 2011; Wacklin et al. 2013; Fallingborg et al. 1989; Leser and Mølbak 2009). The mucosal microbiota will be discussed later in Section 2.3.3 in detail.

Gastrointestinal-tract environments

The thickness of the mucus layer varies along the gastrointestinal tract (GIT), which is important to ensure optimal functionality. Mucus has two layers in both stomach and colon to provide protection against acidic conditions and excessive microbes. In contrast, only a single and discontinuous mucus layer is present in small intestine to allow the efficient absorption of nutrients (Johansson, Larsson, and Hansson 2011).

Mucus contains water (~95%), immunoglobulins (IgA), growth factors, salts, lipids, antibacterial proteins, trefoil factors as well as released epithelial cells (McGuckin et al. 2011), with mucins representing the main structural components. Mucins are heavily glycosylated glycoproteins, which are encoded by the MUC gene family. To

date, 22 human mucins have been identified as either secreted or membrane-bound. Of these, MUC2 is the main secreted mucin in the intestine, whereas MUC5 is the main mucin encountered in the stomach (Table 1) (Johansson, Larsson, and Hansson 2011). The complex mucin-attached glycans do not only serve as an energy source to commensal bacteria, but also act as a binding site for bacterial adhesins (McGuckin et al. 2011). It has been demonstrated that different types and amounts of glycans favor different bacteria as the attachment site to the mucus. The link between glycosylation of the intestinal mucus layer and microbiota composition has been explored in both animal and human studies (Johansson et al. 2013; Sommer et al. 2014; Wacklin et al. 2014; Wacklin et al. 2011).

Once ingested, bacteria enter the GI-tract where they are confronted by the sequential challenges of acid stress in stomach, followed by bile and pancreatic secretions in the small intestine. In the stomach, the pH is less than 3 in healthy adults due to the presence of gastric acid (Fallingborg et al. 1989), exhibiting strong selective pressure which usually results in a low bacterial load (10^1-10^3 cfu/g). A total of 128 phylotypes from 8 phyla were detected in the gastric biopsies taken from 23 adults (Bik et al. 2006). The dominating phylotypes belonged to *Streptococcus* and *Prevotella* (Bik et al. 2006). As reported in this study, the overall bacterial diversity is relatively low, as compared to the about thousand species identified in the colon (Li et al. 2014; Browne et al. 2016). The gastric transit is relatively fast (1h) and some ingested bacteria survive the temporary acidic stress and pass into the small intestine. The stringent acidic conditions no longer exist in the small intestine, where the pH rises significantly from 6.4 in duodenum to 7.3 in ileum (Fallingborg et al. 1989). However, intestinal peristalsis and the intermediate transit time (8h) as compared to stomach and colon (17.5h) still prevents accumulation of microbiota in the small intestine, where 10^4-10^7 bacteria per gram of lumen content are found (Leser and Mølbak 2009). Due to the discontinuous single mucus layer, both luminal and mucosal microbiota can interact extensively with the epithelial cells. In addition, small intestinal microbiota participate in the formation of active, secondary form of bile acids, which are important for host's regulation of lipid and glucose metabolism (Floch 2005; Islam et al. 2011). Most dietary nutrients, i.e. proteins, carbohydrates and fats, are digested and absorbed in the small intestine. Poorly digested non-starch polysaccharides make up the major component of dietary fibre that is metabolized in the large intestine (Floch 2005).

The large intestine serves as a bioreactor for the fermentation of both diet- and host-derived glycans. The pH is 5.7 in caecum and it rises gradually from the right to the left colon with a final mean value of 6.6 (Fallingborg et al. 1989). There are two factors i.e. the more neutral pH and the availability of fermentable substrates, which support a dense (10^{10}-10^{12} cfu/g) and more diverse microbiota (Table 1) (Zoetendal et al. 2012; Lozupone et al. 2012; Rajilić-Stojanović and de Vos 2014). To date, around one thousand gut-related bacterial species have been identified in the large intestine (Browne et al. 2016; Li et al. 2014; Rajilić-Stojanović and de Vos 2014).

Along the GI-tract from stomach to rectum, the decline in the oxygen level shifts the composition of microbiota gradually from facultative anaerobic to obligate anaerobic bacteria (Table 1). Interestingly, oxygen forms a gradient also within the same anatomic location, when it diffuses across the mucosal layer to lumen and thereby enriches certain bacteria along this gradient (Espey 2013). For example, bacteria with respiratory plasticity can tolerate low levels of oxygen for a short time

and have selective growth advantage in the mucosal layer. Some strict anaerobes, for example, *Bacteroides fragilis* and *Faecalibacterium prausnitzii* belong to this group of bacteria (Khan et al. 2012; Baughn and Malamy 2004).

Genetics

It has been proposed that the intestinal microbiota is shaped by both genetic and environmental factors, such as diet (Benson et al. 2010). There are several observations highlighting the influence of genetic factors on the composition of the microbiota. For example, the microbiota is more similar among twins than between two unrelated individuals (Tims et al. 2013; Turnbaugh et al. 2009). However, these studies cannot conclude that the reason accounting for the more similar gut microbiota to be genotype properties without taking into account environmental influences.

Although there are no conclusive genome-wide association studies (GWAS) of microbiota characteristics in humans, polymorphisms attributable to the presence of several single nucleotide polymorphisms (SNPs) have also been shown to be associated with the intestinal microbiota structure and composition. The complexity of mucus results from the highly varying extent and forms of glycosylation of the mucin. Genetic variation in the glycosyltransferase genes expressed in goblet cells explains the diverse glycosylation in different human populations. For instance, *FUT2* is a fucosyltransferase-encoding gene, which determines the so-called secretor-status of the host (Wacklin et al. 2014). Secretors, but not non-secretors, express the ABH and Lewis histo-blood group antigens on mucosal surfaces, including intestinal epithelium. This secretor status has been claimed to explain the different structure and composition of intestinal microbiota in secretors, as compared to non-secretors in healthy adults (Wacklin et al. 2014). In general, non-secretors have been found to exhibit a lower microbial richness and diversity with less bifidobacteria, as compared to secretors (Wacklin et al. 2014; Wacklin et al. 2011).

Host genotype also affects the specific bacterial recognition receptors. For example, nucleotide-oligomerization-domain-2 (NOD2) is an innate immune receptor in the cytoplasm of immune cells. This receptor is involved in the anti-inflammatory pathway in intestine. Studies on mice have revealed that NOD2 has an influence on specific microbiota taxa, as bifidobacteria were absent in NOD2 knock-out mice, displaying severe ileitis (Heimesaat et al. 2014). The risk allele for celiac disease, Human Leukocyte Antigen (HLA)-DQ2, was reported to determine the early intestinal microbiota composition in infants carrying this allele, differentiating them from non-carriers (Olivares et al. 2014). A lower number of *Bifidobacterium* species, and their negative correlations with several genera of Proteobacteria have been found to be associated with HLA-DQ2 high genetic risk allele carriers, in comparison to those subjects without HLA-DQ2/8 alleles (Olivares et al. 2014). Recently, a SNP of apolipoprotein A-V gene, which is the risk allele of metabolic syndrome, was found to be associated with significantly reduced abundance of *Bifidobacterium* independent of age, sex and metabolic syndrome (Lim et al. 2016). In view of the already established links between host genetics and microbiota composition, in the future, an examination of the genetic background should become a routine part of studies investigating host-microbiota interactions in different diseases.

Diet

Diet is considered to be a major external modulator of intestinal microbiota. All major dietary components, carbohydrates, proteins and fats, exert specific and profound effects on the composition of the intestinal microbiota (Salonen and de Vos 2014; Koropatkin, Cameron, and Martens 2012). In general, host-digestible polysaccharides break down in the small intestine to mono- and di-saccharides and the non-absorbed proportion of these simple carbohydrates favors the growth of resident Proteobacteria and Firmicutes such as *Lactobacillales* (Koropatkin, Cameron, and Martens 2012). On the other hand, host-indigestible polysaccharides, mainly dietary fibre, pass down into the colon, where they enrich Bacteroidetes and Firmicutes such as *Clostridiales* (Koropatkin, Cameron, and Martens 2012). However, the microbiota has cross-feeding networks i.e. that bacteria can use metabolites from other bacteria as their energy source and that break-down of complex substrates such as dietary fibre or mucins by specific bacteria can feed also other bacteria. Such cross-feeding and efficient use of available nutrients makes the microbial ecosystem more resilient towards dietary changes. Therefore, day-to-day variation may not be seen in the microbiota composition (Korpela et al. 2014; O'Keefe et al. 2015).

A long-term or a habitual diet has been found to result in a more consistent and profound effect on the microbiota structure, as reviewed in Salonen and de Vos (2014). As shown in several observational studies, even with a consideration of the confounding effect from different ethnicities, the microbiota of inhabitants of western societies can still be significantly different from that of non-western populations (Schnorr et al. 2014; Ou et al. 2013). In general, higher microbial diversity and a reduced abundance ratio of *Bacteroides*/*Prevotella* are observed in people living in close contact with nature and consuming a fibre-based diet, such as the Hadza hunter-gatherers (Schnorr et al. 2014) and native Africans (Ou et al. 2013), as compared to Western populations. These observations indicate that environmental factors, such as habitual diet or some kind of drastic changes of diet, at least to some degree, have greater effect than the host genotype in shaping the microbiota structure. This effect has been also detected in both human and animal intervention studies (O'Keefe et al. 2015; Carmody et al. 2015). For example, the changes of microbiota structure and composition of inbred, transgenic and outbred mice change rapidly and reversibly in response to the fat and sugar levels in the diet (Carmody et al. 2015). Switching from a habitual diet with a high fibre and low fat to high fat and low fibre diet in rural Africans leads to reduced co-occurrence of potential butyrate producers and bacterial taxa utilizing complex carbohydrates (O'Keefe et al. 2015).

2.1.2 The effects of microbiota on host

The intestinal microbiota participates in various digestive processes including salvage of energy to support host growth. Approximately 5% of the daily caloric requirement is estimated to be provided by the fermentation of non-digestible dietary residues, also named dietary fibre (Frayn 2010). The fermentation of dietary fibre is one of the key metabolic pathways and it produces metabolites, such as short-chain fatty acids (SCFA), including acetate, propionate and butyrate (Frayn 2010). In addition, the microbiota participates in synthesizing vitamins such as folate and biotin and affects host iron sensing (Floch 2005; Deschemin et al. 2016).

Furthermore, the microbiota and its products affect both physical and immunological barriers which protect the host against endogenous or exogenous threats (Bischoff et al. 2014).

The role of microbiota in physical barrier of the host

In the intestinal tract, microbiota, gastric juice, pancreatic enzymes, mucus and an epithelium monolayer constitute a physical barrier against pathogens (Viggiano et al. 2015)(Fig. 3A). The epithelial cell monolayer consists of different types of intestinal epithelial cells (IECs) and intercellular junctions, such as tight junctions (TJ). IECs, originating from stem cells present in the crypt, give rise to four main cells types: enterocytes, goblet cells, enteroendocrine cells and Paneth cells (Turner 2009; Umar 2010). Enterocytes are the predominant cell type, making up >80% of all small intestinal epithelial cells (Umar 2010). Goblet cells produce a variety of mucins and trefoil peptides needed for mucus renewal and epithelial growth and repair (Umar 2010) (Fig. 3A). Enteroendocrine cells secrete hormones, which regulate food intake, intestinal transit, release of digestive enzymes, barrier function and immune responses (Furness et al. 2013). Paneth cells secrete defensins, regenerating islet-derived protein 3 (RegIII) and antimicrobial peptides (AMPs) in the villous crypt (Bischoff et al. 2014). All these anti-microbial products and secretory IgA (sIgA) are important in restricting bacterial colonization in crypts to maintain the normal gut epithelium homeostasis (Bischoff et al. 2014).

TJs are considered as an essential structure for controlling intestinal permeability and enterocyte polarity (Turner 2009; Bischoff et al. 2014). They are formed by multiple proteins including occludins and members of the claudin family as the major sealing proteins (Turner 2009; Bischoff et al. 2014)(Fig. 3B). The sealing proteins interact with cytoplasmic proteins, such as zonula-occludens proteins (ZO), functioning as adaptors between the TJ proteins and other proteins such as F-actin within the cells (Turner 2009; Viggiano et al. 2015). Breakdown of this barrier potentially leads to the translocation of luminal antigens, microbiota and their toxic products into the lymphocytic and blood streams (Turner 2009).

Intestinal microbiota itself forms a natural defence barrier by competing with pathogens for ecological niches and nutrients (O'Hara and Shanahan 2006). Furthermore, it processes the molecules necessary for the optimal functioning of the mucosa and strengthens intestinal barrier function. For example, SCFAs, particularly butyrate in the colon, provide an energy source for enterocytes and control the differentiation and proliferation of IECs in healthy or colonic cancer cellular phenotypes (Comalada et al. 2006; Ulluwishewa et al. 2011; Leonel and Alvarez-Leite 2012). Moreover, SCFA can promote the production of mucins and up-regulate the expression of TJ proteins (Peng et al. 2009; Plöger et al. 2012).

The role of microbiota in the immunological barrier of the host

The intestinal epithelium is not only a physical barrier. Its secreted products constitute also an immunological barrier with specialized protective adaptations. Furthermore, in lamina propria (LP), immune cells combat invading microbes and act to eliminate them (Peterson and Artis 2014). Intestinal microbiota, especially commensals, participate in educating the immune system and maintaining the gut homeostasis in the intestine, including both innate and adaptive immunological activities. For example, germ-free animals do not develop normal immune system, as reviewed in Round et al. (2009).

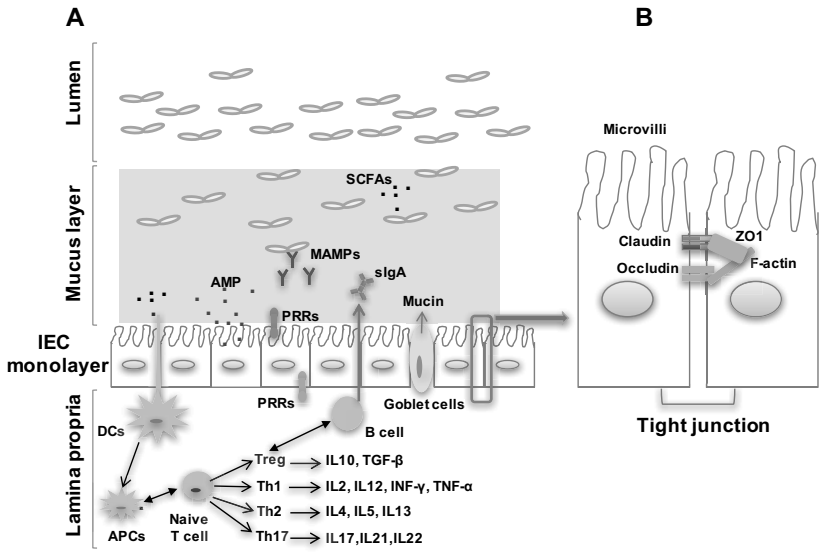

Figure 3. Simple diagram of human small intestinal barrier in healthy individuals. A) Schematic diagram of intestinal barrier. Due to the high ratio of Peyer's patches (not depicted in the figure) in the mucosa, ileum is considered as the main immune sampling site (Bischoff et al. 2014; Kaukinen 2013). B) Tight junctions between intestinal epithelial cells (Turner 2009).

In the lamina propria of small intestine, Peyer's patches (PP) are a specialized gut-associated lymphoid tissue (GALT), where antigen sampling from the gut lumen and the introduction and regulation of immune responses take place. In the small intestine, the sampling of luminal antigens is initiated by specialized epithelium cells, microfold cells (M cells) or dendritic cells (DCs), which can penetrate through the epithelium to sample luminal contents (Fig. 3). An antigen that is presented by M cells can be further passed on to professional antigen presenting cells (APC), including macrophages, B cells and DCs (Furness et al. 2013).

Specialized recognitions between host and microbes are critical initial steps for their interactions. Host recognition of microbe-associated molecular patterns (MAMPs, microbial ligands) is mediated by pattern recognition receptors (PRRs), such as the Toll-like (TLRs) and NOD-like (NLRs) receptors (Table 2, Fig. 3) (Perez-Lopez et al. 2016; Peterson and Artis 2014). These PRRs are expressed in host IECs or immune cells, either on the cell surface or in the cytoplasm (Peterson and Artis 2014; Furness et al. 2013). The expression of PRRs is different in IECs from that in immune cells, and it also differs between the apical and basolateral sides of the IECs (Peterson and Artis 2014; Daig et al. 2000). For example, TLR9 is located on the IECs surface, but resides in the endosomes in immune cells (Peterson and Artis 2014). Moreover, the stimulation of PRRs at only the basolateral side of IECs contributes to the pathogenic activation, while the stimulation at apical sides of IECs is predominantly associated with commensal signaling (Kant et al. 2014; Peterson and Artis 2014). The recognition of MAMPs by TLRs leads a cascade of signalling, which is mediated through multiple adaptor molecules, such as myeloid differentiation primary response gene 88 (MyD88), which in turn activate key regulatory pathways, such as nuclear factor-kB (NF- kB) (Perez-Lopez et al. 2016).

Among all APCs, DCs are the most efficient and they possess enhanced ability to stimulate näive T cells (both $CD4^+$ and $CD8^+$) and subsequently induce the differentiation of antigen-specific T cell subsets, playing an important role in adaptive immunity (Jenkins et al. 2001; Perez-Lopez et al. 2016).

Table 2. Important pattern recognition receptors (PRRs), their recognized microorganisms and localization in human immune cells.

PRR	Recognized microbial structures/molecules	Recognized microorganisms	PRRs in the host immune cell
TLR1	Triacyl lipoproteins	G- bacteria	Cell surface
TLR2	Lipoteichoic acid Lipoproteins Peptidoglycan Lipomannan Zymosan	Bacteria Mycobacteria Yeast	Cell surface
TLR3	Double-stranded RNA	Viruses	Endolysosomal membrane
TLR4	Lipopolysaccharide	G- bacteria	Cell surface Endolysosomal membrane
TLR5	Flagellin	Bacteria	Cell surface
TLR6	Diacyl lipoproteins Lipoteichoic acid Zymosan	G+ bacteria Mycoplasma Yeast	Cell surface
TLR7	sRNA	RNA virus Bacteria	Endolysosomal membrane
TLR8	sRNA	Virus Bacteria	Endolysosomal membrane
TLR9	Unmethylated CpG DNA RNA::DNA	Bacteria Virus	Endolysosomal membrane
TLR10	Unknown	Listeria Influenza A	Cell surface
NOD1	Meso-diaminopimelic acid	Bacteria	Cell cytoplasm
NOD2	Muramyl dipeptide	Bacteria	Cell cytoplasm

G-, Gram-negative bacteria; G+, Gram-positive bacteria; sRNA: single-stranded RNA; RNA::DNA: RNA and DNA hybrids

Microorganisms, especially commensal bacteria, are crucial in the induction of naïve $CD4^+$ T cell differentiation to defence- or tolerance-directed T cell populations (Campbell 2014). T helper 1 (Th1), Th2 and Th17 are defence-directed T cell types, which promote cell mediated immunity, humoral immunity and the recruitment of phagocytic granulocytes, while regulatory T cells (Treg) regulate the balance between these three immune response patterns (Fig. 3) (Campbell 2014; Ohland and Jobin 2015). The differentiated T cell populations together with their secreted cytokines can form pro-inflammatory dominating or anti-inflammatory dominating immune profiles, where a balance in their ratio is believed to initiate tolerance to

normal gut microbiota in early life as a way of maintaining intestinal immune homeostasis (Fig. 3) (Di Mauro et al. 2013).

SCFAs, including acetate, propionate and butyrate, exert extensive effects on the immune system, as reviewed in Vinolo et al. (2011a) and Papato et al. (2014). Importantly, SCFA, particularly butyrate can stimulate the production of IgA (Kim et al. 2016), promote the differentiation and proliferation of regulatory T cells (Smith et al. 2013), and inhibit nuclear factor-kB activation (Vinolo et al. 2011b) and thus act as anti-inflammatory agents. In summary, there is a complex relationship between microbiota, IECs and the immune system and their interactions need to be balanced in order to provide an adequate level of defence while avoiding exaggerated reaction towards commensals to maintain intestinal health.

2.1.3 Intestinal homeostasis in autoimmune diseases

In the past decade, there has been considerable interest about the role of intestinal microbiota in health and disease, since it has been hypothesized that the protective intestinal barrier could be fortified by direct contact with commensal bacteria and their metabolites. Furthermore, intestinal microbiota may provide functional and diagnostic biomarkers that are potentially novel avenues for interventions (Table 3). Common approaches usually rely on high-throughput and other molecular techniques to determine compositional and functional microbial signatures in the comparison of intestinal samples between healthy and compromised subjects (Zoetendal, Rajilic-Stojanovic, and de Vos 2008). In these comparisons, the concept of microbial dysbiosis or imbalance is often utilized (Cheng et al. 2013). This relates to the absence of resilience in the microbial ecosystem resulting in permanent disturbances in the microbiota, in contrast to the stability observed in healthy subjects (Zoetendal, Rajilic-Stojanovic, and de Vos 2008; Jalanka-Tuovinen et al. 2011). On the other hand, microbial dysbiosis has also been linked to an altered composition of the microbiota in comparison to healthy subjects (Round and Mazmanian 2009).

Table 3 Potential biomarkers of GI microbiota in autoimmune disease. Adapted from (Cheng et al. 2013)

Disease	Association with disease	Association with health
CeD	*R.torque* like species	Bifidobacteria
IBD	*R.gnavus, R.torque*	*F.prausnitzii, A.muciniphila*
T1D	-	Bifidobacteria

R= *Ruminococcus*; F= *Faecelibacterium*; A= *Akkermansia*; -indicates that no clear association has been reported.

In general, the intestinal microbiota of subjects with autoimmune diseases has been found to harbour increased numbers of bacteria that are believed to induce inflammation. Figure 4A depicts a general model for the relationship between microbial dysbiosis, compromised barrier function and intestinal inflammation (Cheng et al. 2013). The microbial dysbiosis is manifested as a reduction in the

abundances of protective bacteria, also called symbionts, resulting in a compromised mucosal barrier (Fig. 4B) (Round and Mazmanian 2009). The role of microbial dysbiosis in the pathology of inflammation is not well characterized. It has been proposed that dysbiotic microbiota with increased proportions of pro-inflammatory bacteria or pathobionts can induce non-specific inflammation which may impair the epithelial integrity and promote further inflammation (Fig. 4B) (Kirjavainen et al. 1999; Round and Mazmanian 2009; Pastorelli et al. 2013). Alternatively, as discussed before, different types of microbes can induce the differentiation of different T cell populations, such as Th1, Th2, Th17 and Treg, which are associated with either pro- or anti- inflammatory properties (Fig. 4B). The ratio of Th1/Th2 cell populations is important in the pathogenesis of autoimmune diseases (Th1-polarization) and allergies (Th2-polarization) (Campbell 2014; Rivas et al. 2015). Therefore, microbial dysbiosis may also contribute, together with impaired barrier function, dys-regulated immunity and subsequently to the appearance of mucosal inflammation (Cheng et al. 2013; Ohland and Jobin 2015; Round and Mazmanian 2009). Regardless of the triggering factors, intestinal inflammation may elicit a vicious circle with a progressive disruption of intestinal homeostasis (Fig. 4A) (Cheng *et al.* 2013; Kirjavainen et al. 1999; Ohland and Jobin, 2015). The actual order of the events may vary and, at present, we lack convincing evidence from longitudinal studies, i.e. disease progression being monitored from a healthy status to the disease diagnosis in the same individuals.

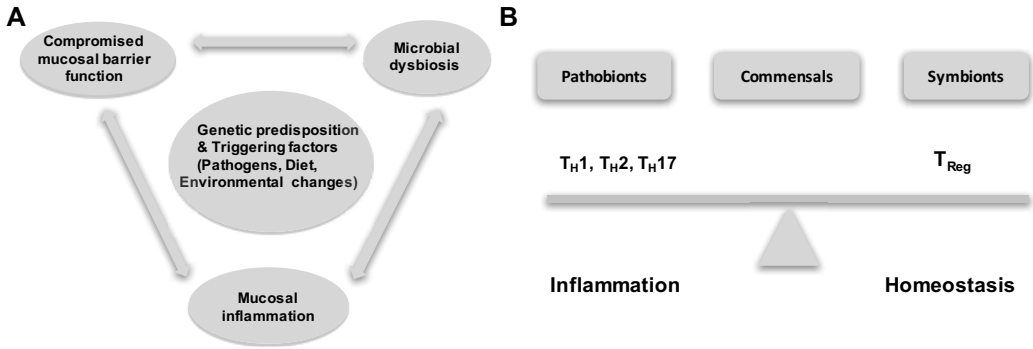

Figure 4. Involvement of microbiota in the regulation of immune homeostasis in autoimmune diseases. A) Model for the relationship between microbial dysbiosis, mucosal barrier function and inflammation, modified from (Cheng et al. 2013). B) Microbiota direct the differentiation of both pro- and anti-inflammatory T cell populations, modified from (Round and Mazmanian 2009).

The studies conducted so far have generated some considerable insights into the role of the intestinal microbiota in major autoimmune diseases such as CeD, IBD and T1D (Table 3).

2.2 The development of a healthy microbiota

The structure and composition of the human intestinal microbiota results from the co-evolution between host and their microbes. Up until now, the majority of studies on intestinal microbiota in health and diseases have focused on fecal microbiota, since feces are accessible in a non-invasive manner and it is easy to obtain. Therefore, this literature review is mostly based on studies utilizing feces as the

study material and thus intestinal microbiota refers to fecal microbiota, unless specified otherwise.

So far, the compositional and functional development of intestinal microbiota in childhood has not been well characterized compared to young infants and adults. Some observational studies suggest that the composition of the microbiota community is fully developed already at 2-3 years of age, although the community structure and diversity in toddlers still differs from that of adults (Bergström et al. 2014; Koenig et al. 2011; Yatsunenko et al. 2012). Furthermore, the development of mucosa-associated microbiota, which is considered to be of crucial importance in determining host-microbiota interactions, is still poorly understood.

2.2.1 Characteristics of healthy intestinal microbiota in adulthood

Taxonomic diversity

The GI-tract, particularly the colon, has the highest density and greatest variety of bacteria in the human body, up to 10^{12} bacterial cells per gram of feces (Sommer and Bäckhed 2013). Bacteria are the most predominant microorganisms living in the gut, representing ~93% of all microorganisms, although a small amount of Euryarchaeota (0.9%, Archaea phylum), fungi (0.1%) and other eukaryotic microorganisms (0.4%) inhabit the human GI-tract (Arumugam et al. 2011). Furthermore, approximately 10^8 virus particles per gram of wet weight have been found in human feces, most of which are phages (Breitbart et al. 2008).

A healthy gut microbiota is dominated by anaerobic bacteria, which outnumber aerobic and facultative anaerobic bacteria by 100- to 1,000-fold (Sommer and Bäckhed 2013). At present, around 1000 species have been identified from the human intestine (Rajilić-Stojanović and de Vos 2014; Li et al. 2014). The latest update was provided by Browne et al., who detected 137 novel culturable bacterial species in healthy fecal samples (Browne et al. 2016). The five most dominant bacterial phyla within the human GI-tract are Firmicutes (19.8-65.6 % of the total microbiota), Bacteroidetes (0.1-64.9%), Actinobacteria (1.1-32.5%), Proteobacteria (0.1-21.2%), and Verrumicrobia (0-8.8%) (Fig.2) (Arumugam et al. 2011). Bacteroidetes and Firmicutes are consistently characterized as the most predominant phyla and they can be detected in virtually all adults, although their relative proportions may vary considerably (Eckburg et al. 2005; Qin et al. 2010; Arumugam et al. 2011). However, at the bacterial species level, there is extensive inter-individual variation in the microbial communities, considerably greater than that observed at the phylum level (Jalanka-Tuovinen et al. 2011; Qin et al. 2010; Rajilić-Stojanović et al. 2012).

In each individual, several hundred species of intestinal bacteria form a resilient ecosystem, characterized by a high level of diversity but temporal stability (Rajilić-Stojanović et al. 2009; Bäckhed et al. 2012; Human Microbiome Project Consortium 2012; Qin et al. 2010). According to a cross-continent cohort study of 39 individuals, the major genera in the gut are *Faecalibacterium* (5.1%), *Bacteroides* (13.9%), *Prevotella* (4.4%), *Bifidobacterium* (4.5%), *Roseburia* (2.6%) and *Collinsella* (1.8%) (Fig.2) (Arumugam et al. 2011). In general, *Bacteroides* is the most abundant genus, but there is very large inter-individual variation (Fig. 5).

Characteristics of this complex ecosystem

In addition to the high diversity, the hallmarks of a normal intestinal microbiota are individual specificity, temporal stability, and conserved key functions (Faith et al. 2013; Muegge et al. 2011; Rajilić-Stojanović et al. 2012; Bashan et al. 2016). The microbiota profiles from the same individual sampled a few days, months or even years apart are more similar to each other than to microbiota profiled at the same time from another individual (Faith et al. 2013; Rajilić-Stojanović et al. 2012; Jalanka-Tuovinen et al. 2011). For example, Faith et al. followed 37 healthy adults and reported that after five years, 60% of the original strains were still present (Faith et al. 2013). Furthermore, Rajilić-Stojanović et al. followed five healthy individuals for up to 12 years, and observed that the overall microbiota profiles were very stable during that time, although there were some variations in the stability of different phyla (Rajilić-Stojanović et al. 2012). The intestinal microbial ecosystem can be influenced by many factors such as genetics and diet. Interestingly, the microbial taxonomic composition generated by 16S rRNA sequencing correlates individually with its functional measurements of microbiota by a metagenomic approach, where 33 mammalian species were compared at one time point, suggesting that it is possible to predict microbial activity from microbiota composition (Muegge et al. 2011). This hypothesis has been confirmed at protein level by metaproteomic analyses of long-term human fecal samples (Kolmeder et al. 2012). Based on this hypothesis, an algorithm named PICRUSt was developed and benchmarked for predicting bacterial functional contents from their taxonomic composition (Langille et al. 2013).

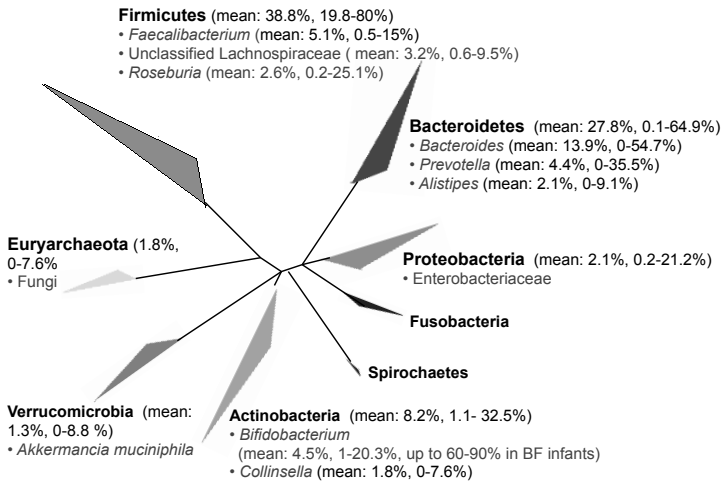

Figure 5. Human intestinal microbiota composition (updated from Cheng et al 2013)

Despite the individual-specific composition, the gut microbiota has extensive functional redundancy, such that the presence of major metabolic pathways was found to be rather similar between individuals (Muegge et al. 2011; Human Microbiome Project Consortium 2012; Turnbaugh and Gordon 2009). These conserved key metabolic pathways are essential for all bacteria, for example ribosome and translational machinery, nucleotide metabolism, ATP synthesis and carbohydrate metabolism (Human Microbiome Project Consortium 2012; Muegge

et al. 2011; Qin et al. 2010; Turnbaugh and Gordon 2009). In addition to the evidence emerging from metagenomics studies, the conserved "core" pathways were also observed at the protein level (Kolmeder et al. 2012). Furthermore, the functionality of microbiota seems to be relatively stable, as indicated by metaproteome profiles of fecal samples from the same individuals taken at different times of one year (Kolmeder et al. 2012). In addition to the functional redundancy in colonic microbiota, some degree of functional redundancy would be expected also in the microbiota found in different parts of the intestinal tract. For example, two mucosa-associated Firmicutes, *Lactobacillales* and *Clostridiales,* carry out digestive functions of harvesting energy from carbohydrates, but perform these tasks in different parts of the intestinal tract, i.e., small and large intestine, respectively (Ohland and Jobin 2015).

Microbiota stratification

The microbial features that are associated with diseases can be characterized by comparing the microbial compositions between normal and disease states. However, the complexity of intestinal microbial ecosystem often leads to a high level of between and within-individual variations, resulting in limited consensus on what should represent a normal or healthy microbiota that can be generalized in all populations (Lozupone et al. 2012). In order to stratify microbiota at the population level, two concepts have been introduced: core microbiota and enterotype (Arumugam et al. 2011; Turnbaugh and Gordon 2009; Sekelja et al. 2011; Wu et al. 2011).

The core microbiota is defined as a set of abundant microbes shared by all or at least most subjects (Turnbaugh and Gordon 2009). The thresholds for the abundance and prevalence of bacteria to be included in the core still vary between the studies. For example, in some individuals, common commensals such as *Faecalibacterium prausnitzii* and *Roseburia intestinalis* may have low abundance and have been excluded from the core in one report (Turnbaugh and Gordon 2009), but included in another study (Jalanka-Tuovinen et al 2011). The healthy core in a Finnish study which examined nine subjects contained around 280 phylotypes belonging mainly to two groups of Firmicutes: *Clostridium* clusters IV and XIVa (Jalanka-Tuovinen et al. 2011). Phylotypes assigned to these two Firmicutes have also been observed as core members by other investigators (Qin et al. 2010; Sekelja et al. 2011). Therefore, the core microbiota approach is clearly dependent on the study designs. If one wishes to include different study populations with varying ages and nationalities, then defining what represents the core microbiota will become even more challenging. The taxonomic core microbiota determined in U.S. children will be discussed in Section 2.2.2 and in publication III of this thesis.

Unlike the core microbiota approach, which includes microbes according to their abundance and prevalence, an enterotype analysis focuses on the functional interactions between microbes such that an enterotype refers to the co-existence of certain microbes which might contribute to the same function at a population level (Arumugam et al. 2011). In the EU MetaHIT consortium project, Arumugam et al. found three enterotypes including *Bacteroides*, *Prevotella* and *Ruminococcus* as the main driving genera in the bacterial communities, while the US Human Microbiome Project (HMP) confirmed only two of them (*Bacteroides and Prevotella*) (Arumugam et al. 2011; Wu et al. 2011). Interestingly, these two enterotypes were found to correlate with habitual diets (Wu et al. 2011). The difference in the number of enterotypes revealed by these two studies may be

attributed to the geographic variation between EU and US or differences in the microbiota assessment platforms. Furthermore, additional enterotypes might become evident when children and other populations are included in the analyses. For example, the clear differences in microbiota composition between infants and adults may indicate the presence of age-related novel enterotypes, while the clear difference between adults from Malawi or Venezuela and US (Yatsunenko et al. 2012) may indicate the presence of novel geography-enterotypes in addition to those already described. However, the concept of "enterotype" was challenged by Knights et al. (2014), where the majority of the analysed data in the microbiome studies demonstrated a continuous gradient of dominant taxa rather than discrete enterotypes.

2.2.2 Compositional changes of microbiota in childhood and influencing factors

The colonization, diversification and establishment of human intestinal microbiota has been considered as a step-wise process, which is influenced by a number of factors (Rautava, Luoto, et al. 2012; Nylund et al. 2014). The major introduction of both microbial and dietary antigens happens during infancy (0-3y) and it is influenced by many perinatal events and early life nutritional changes, resulting in major colonization and diversification of intestinal microbiota. This is reflected in the rapid increase in the diversity and stability of the microbiota that occurs during the first year of life (Fig. 6) (Rautava, Luoto, et al. 2012; Nylund et al. 2014). In general, the intestinal microbiota is less stable in early life than in adulthood (Fig. 6) (Yatsunenko et al. 2012). Therefore, early life is considered to be a critical period for developing tolerance towards commensals and maturating immune system (Di Mauro et al. 2013; Isolauri 2012; Rautava, Luoto, et al. 2012) (Fig. 6).

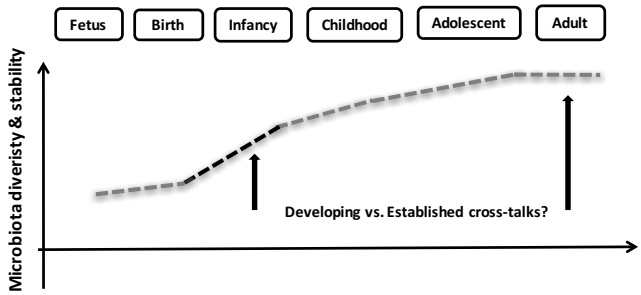

Figure 6. Development of tolerance towards commensal bacteria. Intestinal microbiota diversity reflects both how many microbes are present (richness) and their distribution pattern (evenness) in the ecosystem, while stability reflects the structural configuration of the ecosystem over time. Both diversity and stability may be reflected in the host-microbe and environment-microbe interactions. As discussed in Section 2.2.1, both diversity and stability reach their highest levels in adulthood, indicating that by that time, the host-microbe-environment cross-talk may also be established.

Prenatal period and mode of delivery

Traditionally, the human fetus is considered to be free from microbes and bacterial colonization is known to start during or soon after the birth of the newborn. In the past decades, this consensus has been challenged by the detection of bacterial DNA signatures in placenta and amniotic fluid in healthy individuals (Keski-Nisula et al. 1997; Satokari et al. 2009; Aagaard et al. 2014). Moreover, in healthy neonates born

by Cesarean section, live bacteria have been found in the umbilical cord blood and meconium (Jiménez et al. 2005; Jiménez et al. 2008). It may be speculated that the presence of microbes *in utero* is not necessarily linked to any pathological consequences, but rather to the education of the fetal's immune system. The genera that have been detected in meconium using culture analysis are *Enterococcus* and *Staphylococcus* (Jiménez et al. 2005; Moles et al. 2013). Furthermore, the mother-to-child microbial transmission has been confirmed by detecting *Bifidobacterium lactis* alone or mixed with *Lactobacillus rhamnosus GG* in placenta, after oral consumption of these two bacteria by their mothers in a double blinded randomized trial (Rautava, Collado, et al. 2012). Therefore, it has been claimed that the first inoculum is inherited from the mother (Jiménez et al. 2005; Satokari et al. 2009). However, the knowledge of maternal symbiont transmission is limited, due to the sterile womb paradigm, which poses ethical limitations in addition to technical difficulties such that it is difficult to collect samples from healthy pregnant women before delivery.

Although the initial exposure to microbes could be *in utero*, the main microbial colonization is known to begin during birth, when the infant becomes exposed to the extra-uterine environment (Rautava, Luoto, et al. 2012). During the subsequent colonization process, new microbes are introduced through a variety of environmental contacts. Facultative anaerobic bacteria e.g. Enterobacteria, are replaced gradually by strict anaerobic bacteria, such as *Bifidobacterium*, *Clostridium*, and *Bacteroides* (Arrieta et al. 2014; Matamoros et al. 2013; Weber and Polanco 2012). The favourable microbiota seems to result from the beneficial co-evolutionary relationship with the infants born vaginally in full term, who exclusively drink breast milk during the first few months (Collado et al. 2012; Cheng et al. 2013). In contrast, the microbiota in Cesarean-delivered infants have low levels of *Bacteroides* and bifidobacteria and high level of *Clostridium spp.* (Grönlund et al. 1999; Adlerberth et al. 2007; Palmer et al. 2007; Kuitunen et al. 2009). Such differences in microbial composition can even be detected in the infants of one year old (Grönlund et al. 1999; Adlerberth et al. 2006; Penders et al. 2006).

Early-life Nutrition

Maternal nutrition plays important role in fetal development, which subsequently affects the fetal intestinal microbiota. After birth, breast milk is the major or even exclusive food for infants, containing numerous bioactive compounds, which also act as modulators for the infant intestinal microbiota development, as reviewed in Rautava (2016).

Human milk oligosaccharides (HMO) are important modulating components for the infant microbiota. The number of HMOs in human breast milk is much higher than that in cow milk, as reviewed in Nylund et al. (2014). These oligosaccharides can boost the growth of bifidobacteria including some *Bifidobacterium longum* strains, and a higher level of these bacteria is thought to represent a specific microbial signature of the infant gut (Schell et al. 2002; Sela et al. 2008; Zivkovic et al. 2011; Satokari et al. 2002). In 1905, Tissier (1905) described the distinctive microbiota of breast-fed (BF) infants, dominated by *Bifidobacterium bifidum*. The dominant role of bifidobacteria in BF infants still seems valid today. Instead, formula-fed (FF) infants seem to harbor more mixed-type microbiota and harbor e.g. high numbers of *Bacteroides* in addition to bifidobacteria (Harmsen et al. 2000), although the levels of bifidobacteria do not differ significantly between BF

and FF infants, summarized in Adlerberth and Wold (2009). Interestingly, supplement with prebiotics in formula-feeding leads to the enrichment of bifidobacteria and lactobacilli, which exert proctective effects to the infant, as reviewed in Bertelsen, Jensen, and Ringel-Kulka (2016). For example, *Bifidobacterium longum* and *Lactobacillus rhamnosus GG* have been reported to alleviate the symptoms during GI-tract infections (Guarino et al. 2014).

Breast milk microbiota has been recently characterized as an individual-specific microbial ecosystem, distincting to that of the other human body compartments (Hunt et al. 2011; Cabrera-Rubio et al. 2012). *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Bifidobacterium* spp. have been detected in breast-milk by both cultural dependent and independent approaches, as reviewed in Gomez-Gallego et al. (2016). Furthermore, common core microbiota comprising seven genera has been proposed by Hunt et al. (2011) and Jiménez et al. (2015). Among these seven core genera, *Staphylococcus* and *Streptococcus* were revealed in both studies (Hunt et al. 2011; Jiménez et al. 2015). Breast milk microbiota may affect the infant intestinal microbiota development, since the same groups of bacteria, including *Bifidobacterium* spp. have been consistently detected in maternal feces, breast milk and the infant gut, as reviewed by both Gomez-Gallego et al. (2016) and Rautava S (2016). However, the routes of transmission between mother and child are still not fully understood and currently under intensive investigation.

The main diversification of the infantile microbial population starts after weaning from breast milk. Bifidobacteria predominate during the first few months, especially in BF infants (Roger and McCartney 2010). The expansion of the phyla Bacteroidetes and Firmicutes (including the genera *Lachnospira*, *Clostridium* and *Ruminococcus*) is associated with weaning and the introduction of solid foods at about 4-6 months (Fallani et al. 2011; Koenig et al. 2011). In contrast, *Bifidobacterium* spp. and *Enterobacteria* species abundance starts to decrease (Fallani et al. 2011). The conversion to an adult-type microbiota has been gradually achieved by 1-2 years of age (Palmer et al. 2007; Mackie, Sghir, and Gaskins 1999). However, the actual age of the maturation and stabilization of the intestinal microbiota has not been investigated sufficiently in long-term studies. For example, the turning point for *Bifidobacterium* spp. from infantile to adult-type profile has not been specified (Shkoporov et al. 2008). In general, the post-weaning diversification of intestinal microbiota could be rapidly shifted by a fibre-rich diet, resulting in region-specific microbial signatures in rural African children compared to their European counterparts (Filippo et al. 2010). More specifically, rural African children had a significantly higher level of SCFAs in feces, enriched Bacteroidetes with abundant *Prevotella* and *Xylanibacter*, and on the other hand the depletion of Firmicutes and lower level of *Enterobacteriaceae* (*Shigella* and *Escherichia*). These findings support the co-evolved relationship between a fibre-rich diet and specific intestinal microbial signatures.

Antibiotics

Infants, toddlers and young children are at a high risk of suffering infections. In Finland, high morbidity is observed within the first year of age, with an average of 4 to 7 ear or respiratory tract infections, often accompanied by a couple of enteric infections (Peltola 2012). Antibiotics are among the most commonly prescribed drugs for young children.

In general, antibiotic treatments are considered as a major perturbation of intestinal microbiota, resulting in reduced bacterial diversity, altered microbial community structure and composition (Korpela et al. 2016; Rehman et al. 2012; Dethlefsen and Relman 2011). These effects may be transient or persistent, depending on both the type and duration of antibiotic therapy. Korpela et al. reported that one course of macrolide therapy exerted a much stronger effect than penicillin on both overall microbial richness and specific microbiota abundance (Korpela et al. 2016). After macrolide administration, microbial richness did not recover to the control level even after two years, whereas no significant reduction in richness was found in children with penicillin treatment at six months' post-treatment (Korpela et al. 2016). The depletion of Actinobacteria as well as increased abundance of Baceroidetes and Proteobacteria due to macrolides effects persist for six months, while penicillin did not show similar results (Korpela et al. 2016). Oral administration of vancomycin achieved similar results with macrolide treatment in adults, such as reduced diversity and increased abundance of Proteobacteria, while no significantly reduced diversity was observed in adults treated with amoxicillin (Vrieze et al. 2014). Moreover, repeated treatments with ciprofloxacin has been reported to lead to incomplete recovery of microbiota to baseline, with an altered overall composition representing a new ecologically stable state (Dethlefsen and Relman 2011).

Environmental biodiversity

Biodiversity is the measurement of variety of all living organisms in the environment, including plants, animals and microorganisms. A comparison of the biodiversity can be conducted within species, between species and at the level of the whole ecosystem. In microbial ecology, recent advances in biodiversity research have focused on genetic diversity of populations and species, such as the assessment of 16S rRNA gene sequences or whole (meta) genome based high-throughput intestinal microbiome research (Muegge et al. 2011; Human Microbiome Project Consortium 2012; Korpela et al. 2016).

Environmental biodiversity, especially environmental microbiota diversity, has been proposed to be the link between environmental and lifestyle changes and human diseases, as postulated in the so-called hygiene or biodiversity hypothesis (von Hertzen, Hanski, and Haahtela 2011). According to this hypothesis, the lack of microbial exposure to natural and agriculture environments, e.g. farm, is thought to lead intestinal microbiota dysbiosis and skew the immune response against common and innocuous environmental antigens, increasing the risk of immune-mediated diseases which will be discussed in details in Section 2.3.3 (Mutius et al. 2010; Haahtela et al. 2013; von Hertzen, Hanski, and Haahtela 2011). Today, processed food contains few microbes and the exposure to microbes via the consumption of fermented foods is also low in Western societies (Logan, Katzman, and Balanzá-Martínez 2015). Furthermore, common practices such as the use of household disinfectants and pre-filtering incoming air through automated ventilation contribute to today's excessively hygienic life-style.

There are a number of factors that could affect intestinal microbiota development from fetus to adulthood, such as birth time, family size and duration of lactation (Fig. 7). These have been extensively reviewed recently (Rautava, Luoto, et al. 2012; Putignani et al. 2014; Spor, Koren, and Ley 2011; Yatsunenko et al. 2012).

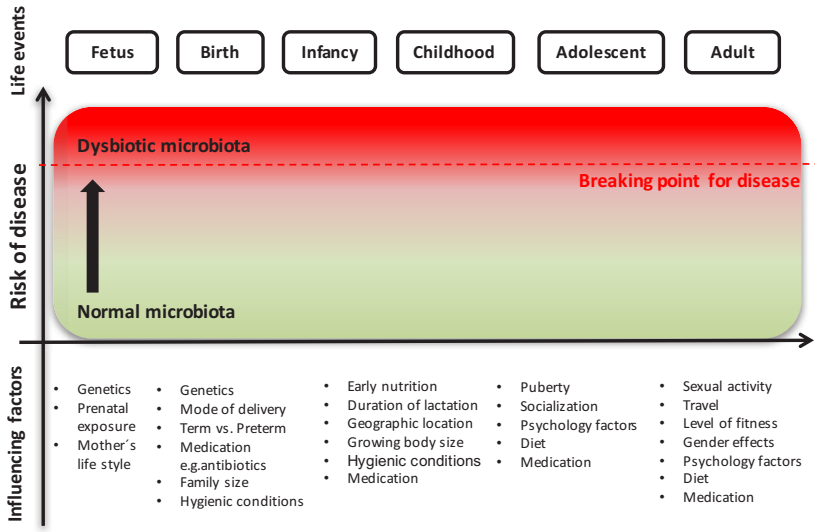

Figure 7. Host-life events that influence intestinal microbiota development. Throughout the life span, different internal and external factors can accumulatively affect host health either directly or by affecting the microbiota. Accumulated negative effects from influencing factors can interfere with the balance of normal microbiota, which may gradually lead to a dysbiotic microbiota and increased risk of disease development.

Core microbiota in childhood

In childhood, less stable intestinal microbial ecosystem often refers to less resilience and more sensitivity towards both internal and external perturbations, such as host physiology development events, infections and medications (Fig. 6 and Fig. 7). The accumulation of different perturbations can result in extensive variations in microbiota composition within and between individuals in children as compare to adults (Yatsunenko et al. 2012). Therefore, it is even more challenging to stratify the intestinal microbiota in children than in adults, and identification of the core microbiota might be a good approach for characterizing the development of intestinal microbiota in children. There are rather few studies that have investigated the core microbiota in children (Table 4).

2.2.3 Microbial dysbiosis in early life and associations with diseases

As depicted in Figure 7, an accumulation of different perturbations might result in (individual-specific) dysbiosis of microbiota. Subsequently, as discussed in Section 2.1.3, microbial dysbiosis can lead to altered mucosal homeostasis and immune-dysregulation, which has been observed in numerous pediatric diseases, including metabolic diseases, allergies, autoimmune diseases, even brain disorders (Renz, Brandtzaeg, and Hornef 2012; Rook, Lowry, and Raison 2015). For example, a microbial dysbiosis associated with Caesarean section might increase the risk of allergy (Guibas et al. 2013) and autoimmunity (Bonifacio et al. 2011). Antibiotics-related changes in intestinal microbiota can influence subsequent microbial

Table 4. Summary of common core microbiota studies in healthy pediatric population.

Healthy study population	Sample type and sampling	Microbiota assessment	Definition for the core microbiota	Main findings	Reference
USA (N=5, 2Y, 4Y, 6Y, 8Y, 10Y) These children are siblings	Feces Longitudinal sampling taken daily for 26 days	Pyrosequencing on 16S rRNA V3-V5 region	Common core microbiota contains OTUs that have at least 0.05% abundance in 95% of all samples	Common core contains 12 OTUs,which represent 32.0-57.9% of sequences from the total microbiota and belong to Lachnospiraceae (6 OTUs), Ruminococcaceae (1 OTU), *Subdoligranulum* (1 OTU), *Dorea* (1 OTU), Clostridum XVIII (1 OTU), *Anaerostipes* (1 OTU) and *Blautia* (1 OTU)	(Schloss et al. 2014)
Netherlands (N=61, 2-18Y)	Feces Weekly samples for 6 weeks and then one follow-up sample after 18 months	Pyrosequencing on 16S rRNA V1-V3 region	Common core microbiota contains OTUs present in more than 80% of all individuals	Age-independent common core is dominated by the phylum Bacteroidetes, with the genera *Bacteroides* and *Alistipes* having the highest average abundance.	(de Meij et al. 2016)
USA (N=22, 11-18Y)	Feces 1 sample/subject	16S rRNA gene full-length microarray	Common core microbiota contains bacterial species shared by all individuals	Common core contains 46 bacterial species, which belong to genus *Ruminococcus* (27 species), *Faecalibacterium* (4 species) and *Roseburia* (4 species), other genera within the class *Clostridia (9 species) and Bacilli* (2 species) Core microbiota constitutes on average 24.8% (17.5-34.4%) of the total microbiota.	(Agans et al. 2011)

colonization, leading to the loss of colonization resistance and providing opportunities for pathogens such as rotavirus and in rare cases even *Clostridium difficile* to cause infection (Morgun et al. 2015; Viggiano et al. 2015). Furthermore, antibiotic treatments, particularly their overuse, increase the prevalence of antibiotic-resistant bacterial strains in the community; which is now one of the main threats to public health (Cotter, Ross, and Hill 2013; Moore et al. 2013). In addition, antibiotics can induce gut alterations by directly effecting the host epithelium and mitochondrial functions, such as decreasing the host cell viability (Morgun et al. 2015; Moullan et al. 2015). The use of antibiotics has recently been associated with the development of CeD and excessive weight gain in children with microbial dysbiosis as one plausible explanation (Morgun et al. 2015; Mårild et al. 2013; Korpela et al. 2016). Furthermore, repeated antibiotic treatments in childhood have been linked with an increased risk for the development of Crohn´s disease, but not ulcerative colitis (Virta et al. 2012). The link may indicate the role of either antibiotics or infections or both in the development of Crohn´s disease.

The loss of microbial biodiversity in food together with the reduced exposure to environmental microbes in early life in today's Western life-style may have profound effects on human life, such as delayed maturation of the immune system and a high risk for suffering infections (Haahtela 2014). Furthermore, the loss of intestinal microbial biodiversity is typical in Western population can only be reversed by dietary means in a single generation, but not over several generations, as indicated in mice (Sonnenburg et al. 2016). In addition, it has been suggested that 50-60% of intestinal microbial genera from healthy individuals are spore-producing bacteria, which have a better possibility of surviving a prolonged exposure to some common disinfectants than non-spore forming commensals (Browne et al. 2016). This finding highlights the possible transmission route of commensals between humans and the environment. On the other hand, the extensive use of disinfectants in modern life confers a survival advantage on the spore-forming pathogens such as *Clostridium difficile*, which might cause severe infectious diseases. Emulsifiers and other detergent-like molecules are ubiquitous components of processed Western food and their extensive use has been considered one potential reason for increased incidence of chronic inflammatory diseases such as metabolic syndrome (Chassaing et al. 2015). A possible mechanism has been proposed that emulsifiers in foods could disturb the production of SCFAs and bile acid metabolism, leading to disrupted host-microbe interactions and low grade inflammation (Chassaing et al. 2015).

2.3 Pediatric celiac disease and microbiota

2.3.1 Celiac disease

Celiac disease is a chronic immune-mediated disease characterized by small intestinal inflammation introduced by an intolerance to gluten or related prolamins (Ivarsson et al. 2013). The incidence of CeD in Western countries, such as Europe and USA is increasing, with an approximate frequency of 1% in the general population (Mustalahti et al. 2010; Fasano et al. 2003). The disease prevalence varies extensively within Europe, with the highest prevalence encountered in Finnish adults (2.4%) and the lowest in Germany (0.3%) (Mustalahti et al. 2010). Active CeD patients typically have mucosal injury with villous atrophy affecting nutrient absorption as well as lymphocyte levels in the LP (Verdu, Galipeau, and

Jabri 2015). The typical symptoms of CeD are diarrhea, malabsorption or even malnutrition in severe cases (Green and Jabri 2006). Moreover, extra-intestinal symptoms including dermatitis herpetiformis may also occur. Adherence to a strict gluten-free diet is currently the only effective treatment for CeD (Green and Jabri 2006).

CeD occurs in genetically predisposed individuals of all ages with the initial symptoms appearing at different times in different individuals - from infancy (after introduction of gluten-containing food) to old age (Aronsson et al. 2015). The progression of the disease is known to be influenced by both genetic and environmental factors (Fig. 8). Association studies have revealed strong genetic effects. Human Leukocyte Antigen (HLA) class II molecules (HLA -DQ2 or HLA-DQ8) are the major genetic risk factors predisposing individuals to CeD, explaining about 40% of the genetic variance (Trynka et al. 2011). Other 39 non-HLA loci, including *FUT2* account for about 14% of the genetic variance (Trynka et al. 2011; Parmar et al. 2012). Moreover, *FUT2* non-secretor status was reported to have increased risk of CeD (Parmar et al. 2012). However, not all genetically predisposed individuals will eventually develop CeD, suggesting the attribution from other unknown genetic factors and/or environmental factors are important in CeD pathogenesis (Trynka, Wijmenga, and van Heel 2010; Green and Jabri 2006; Olivares et al. 2014; Decker, Hornef, and Stockinger 2011; Ivarsson et al. 2013; Canova et al. 2014).

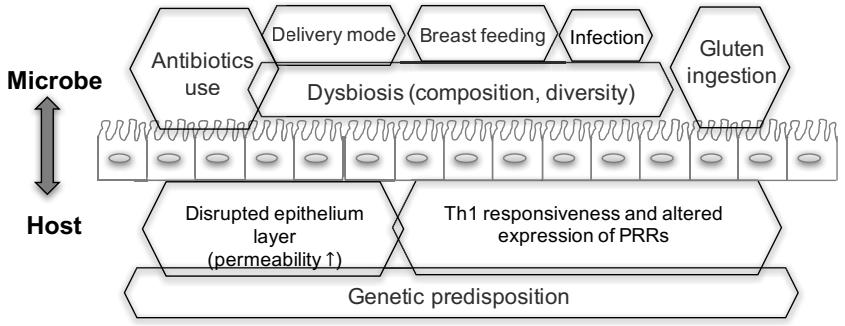

Figure 8. Influencing factors on celiac disease onset. HLA-DQ2/DQ8, infections and gluten ingestion directly affect epithelium integrity and immunological regulations, which subsequently may predispose to Th1-type immunity and disease onset (Green and Jabri 2006). Moreover, HLA-DQ2 (Olivares et al. 2014) and other environmental factors, such as antibiotics (Mårild et al. 2013), delivery mode (Decker, Hornef, and Stockinger 2011), infections (Canova et al. 2014) and breast-feeding (Ivarsson et al. 2013), can promote the disease development via dysbiotic colonization process during infancy.

2.3.2 Pediatric celiac disease associated microbiota

As discussed in Section 2.1.2, the intestinal microbiota is considered to be an important factor contributing to the homeostasis of the gut epithelium, which may play an essential role in the CeD onset (Fig. 8) (Verdu, Galipeau, and Jabri 2015; Palma et al. 2012). Several research groups have compared the microbiota composition in active CeD patients, gluten-free diet treated individuals and healthy controls in paediatric populations by examining their biopsy samples (Table 5).

In general, as compared to healthy controls, increased abundance and/or prevalence of facultative bacteria, such as *Staphylococcus* and Proteobacteria,

particularly *E.coli*, seem to be a common feature of duodenal mucosa-associated microbiota in active CeD patients (Nadal et al. 2007; Collado et al. 2009; Sánchez et al. 2013) and are reduced after GFD treatment (Collado et al. 2009; Sánchez et al. 2013) (Table 5). The increased levels of facultative bacteria may be associated primarily with intestinal inflammation. Interestingly, a shift from predominantly anaerobic microbiota towards a facultative anaerobic community has been frequently observed in IBD subjects with active disease as well, as reviewed in (Mukhopadhya et al. 2012; Rigottier-Gois et al. 2013). One proposed mechanism behind the shift is so-called "oxygen hypothesis", which suggests that inflammation increases the oxygen level in mucosa, thereby favouring facultative anaerobes (Mukhopadhya et al. 2012; Rigottier-Gois et al. 2013). Likewise, in CeD inflammation may drive microbiota dysbiosis. Furthermore, increased duodenal mucosal microbial diversity and total bacterial counts have been observed in active CeD patients as compared to healthy controls (Nadal et al. 2007; Schippa et al. 2010). Furthermore, microbiota similarity between individuals is higher in subjects with active CeD than in CeD subjects on GFD or in healthy controls (Schippa et al. 2010). Such microbiota features may also reflect inflammation driven microbial dysbiosis, but it is often not possible to distinguish between the effects of mucosal healing and the ceasing of inflammation from those induced by changes in the diet.

GFD is the only effective treatment for CeD. The withdrawal of gluten containing cereals from the diet was shown to lead to decreased consumption of dietary fibre in adults (De Palma et al. 2009). Several studies have reported the microbiota changes due to GFD treatment (Table 5). The changes in microbiota composition could be caused not only by the withdrawal of gluten but also by the low fibre intake. Comparison of CeD subjects on GFD and healthy controls has provided some insights into the GFD induced changes in microbiota, although the observed differences in microbiota may also reflect incomplete recovery of microbiota from severe inflammation-driven microbial dysbiosis after the implementation of GFD. For example, decreased total counts in mucosal bacteria, contributed mainly by decreased counts of *Bifidobacterium* and *Lactobacillus,* in GFD individuals as compared to healthy controls have been reported (Nadal et al. 2007). Di Cagno et al. (2011) also reported increased diversity of mucosal Eubacterial community in GFD treated groups than that in HC, indicating the rearrangement of the species within this group. Bonder et al. (2016) evaluated the effect of GFD on microbiota in 21 healthy individuals before and after four weeks GFD. Short-term GFD diet implementation had only a moderate effect on the abundance of several bacterial taxa, such as reduced abundance of *Veillonellaceae*, but more profound effects on the microbiota activities, mainly starch degradation (Bonder et al. 2016). GFD did not affect gut inflammation related biomarkers such as fecal calprotectin (Bonder et al. 2016). The result suggested that GFD does not have a significant contribution to the CeD-associated microbial dysbiosis.

Although mucosal microbiota affects the host by directly communicating with immune cells and IECs, luminal microbiota also can modulate host immune system via metabolites as well as MAMP and toxin production. Microbial dysbiosis has also been consistently observed in CeD-associated fecal microbiota (Table 6). In some studies, similar patterns of microbial dysbiosis have been observed for CeD-associated fecal and duodenal mucosal microbiotas i.e. increase in facultative anaerobes and diversity (Sanz et al. 2007; Collado et al. 2009; Sánchez et al. 2012). Interestingly, comparing to healthy controls, significantly reduced amounts of IgA-coated bacteria was detected in both untreated and treated

Table 5. Studies on mucosal microbiota in pediatric celiac disease

Study population	Sample	Microbiota assessment	Main microbiota findings		Reference
			Disease effect (microbial differences as compared to HC)	**GFD effect (microbial differences as compared to CeD or HC)**	
CeD: N=20, mean age 5.1 years CeD-GFD: N=10, mean age 5.6 years HC: N=8, mean age 4.1 years	Duodenal biopsy	FISH and FC	**CeD vs. HC**: total bacteria count ↑, *Bacteroides-Prevotella* counts ↑, *E. coli* counts ↑ **(CeD&CeD-GFD) vs. HC:** (*Lactobacillus-Bifidobacterium)/* (*Bacteroides-E. coli)* ↓	**CeD-GFD vs. HC**: total bacteria ↓, *Bifidobacterium*↓, *Lactobacillus* group↓, *F. prausnitzii*↓, *E. rectale* ↓ **CeD-GFD vs. CeD**: total bacteria ↓, *Bifidobacterium*↓, *Lactobacillus* group↓, *E. rectale*↓	(Nadal et al. 2007)
CeD: N=25, age 4.7-5.1 years CeD-GFD: N=8, age 4.8-5.3 years HC: N=8, age 3.8-4.1 years	Duodenal biopsies	qPCR	**CeD vs. HC**: *Staphylococcus* counts ↑, *C.coccoides* prevalence ↓ **(CeD & CeD-GFD) vs. HC**: *Bacteroides* counts↑, *C. leptum* counts ↑, *E. coli* counts ↑, *Bifidobacterium* counts↓, *Lactobacillus* counts ↑	**CeD-GFD vs. HC**: *Lactobacillus* prevalence ↓ **CeD-GFD vs. CeD:** *Staphylococcus* counts ↓, *Lactobacillus* counts ↓, *E. coli* counts ↓, *Lactobacillus* prevalence ↓, *A. mucinophilia* prevalence ↓	(Collado et al. 2009)
CeD: N=33, mean age 5.9 years CeD-GFD: N=17, mean age 7.5 years HC: N=18, mean age 3.2 years	Duodenal or jejunal biopsy	16S rDNA sequencing, SEM, culturing	In general, no marked difference between the groups. However, biopsies from CeD patients born during the Swedish CeD epidemic (1985-1996) were enriched with rod-shaped bacteria tentatively identified as *Clostridium* spp., *Prevotella* spp. and *Actinomuces graevenitzii.*		(Ou et al. 2009)
CeD: N=20, mean age 8.3 years CeD-GFD: N=20, mean age 8.3 years HC: N=10, mean age 11.7 years	Duodenal biopsy	TTGE	**CeD vs HC**: Bacterial diversity ↑	**CeD-GFD vs. CeD**: Bacterial diversity ↓	(Schippa et al. 2010)

CeD: N=20, mean age 4.3 years CeD-GFD: N=12, mean age 4.6 years HC: N=8, mean age 4.2 years	Duodenal biopsy	DGGE	**CeD vs. HC**: Bifidobacterial diversity ↑, *B. adolescentis* ↑, *Bifidobacterium animalis subsp. Lactis*↑, Lactic acid bacteria diversity ↓ **(CeD & CeD-GFD) vs. HC**: *Bacteroides* diversity ↓, *Bifidobacterium* diversity↑	**CeD-GFD vs. CeD**: Lactic acid bacteria diversity ↑	(Sánchez et al. 2010)
CeD-GFD: N=19, median age 9.7 years HC: N=15, median age 10.4 years	Duodenal biopsy	PCR-DGGE		**CeD-GFD vs. HC**: Eubacteria diversity↑	(Di Cagno et al. 2011)
CeD: N=10, mean age 8.8 years HC: N=10, mean age 8.7 years	Duodenal biopsy	qPCR	No marked difference found in total bacteria counts, *Bifidobacterium spp.*, *Staphylococcus-Prevotella-Porphyromonas*, *Bacteroide. fragilis*, *Streptococcus* and *Lactobacillus* among case-control groups		(Kalliomäki et al. 2012)
CeD: N=8, mean age 3.75 years HC: N=5, mean age 7.2 years	Duodenal biopsy	16S rDNA sequencing	**CeD vs. HC**: *Prevotella* spp. *& Streptococcus* spp. ↓		(Nistal et al. 2012)
CeD: N=32, mean age 5.1 years CeD-GFD: N=17, mean age 5.9 years HC: N=8, mean age 6.9 years	Duodenal biopsy	16S rDNA sequencing, Culturing	**CeD vs. HC:** Firmicutes ↓, Proteobacteria↑	**CeD-GFD vs. CeD:** Actinobacteria↓, Firmicutes↑, Proteobacteria ↓,	(Sánchez et al. 2013)

CeD – active celiac disease subjects; CeD-GFD - celiac disease subjects treated with gluten free diet; HC – healthy controls

CeD vs. HC: microbiota findings in active CeD patients, as compared to HC. The same designation was used for the abbreviations: CeD vs. CeD-GFD and CeD-GFD vs. HC

FISH - fluorescent *in situ* hybridization; FC - flow cytometry; DGGE - denaturing gradient gel electrophoresis; TTGE – temporal temperature gradient gel electrophoresis

SEM – scanning electron microscopy; (q)PCR – (quantitative) polymerase chain reaction

Table 6. Studies on fecal microbiota in pediatric celiac disease

Study population	Microbiota assessment	Main microbiota findings		Reference
		Disease effect (microbial differences as compared to HC)	**GFD effect (microbial differences as compared to CeD)**	
CeD: N=10, mean age 2.3 years HC: N=10, mean age 2.0 years	DGGE	**CeD vs. HC**: Bacterial diversity ↑, Bifidobacterial diversity ↓, *Lactobacillus casei*↓, *Lactobacillus curvatus*↑		(Sanz et al. 2007)
CeD: N=30, age 4.7-5.1 years CeD-GFD: N=18, age 4.8-5.3 years HC: N=30, age 3.8-4.1 years	qPCR	**(CeD & CeD-GFD) vs. HC**: Total bacterial count↑, *Bacteroides* counts↑, *C.leptum* counts ↑, *Bifidobacterium* counts↓ **CeD vs. HC**: *E. coli* counts ↑, *Staphylococcus* counts ↑	**CeD-GFD vs. CeD:** *Staphylococcus* counts ↓ **CeD-GFD vs. HC**: *Lactobacillus* counts ↑	(Collado et al. 2009)
CeD: N=24, mean age 5.5 years CeD-GFD: N=18, mean age 5.5 years HC: N=18, mean age 5.3 years	FISH and FC	**(CeD & CeD-GFD) vs. HC**: IgA-coated bacteria counts ↓ **CeD vs. HC**: *Bifidobacterium* counts ↓, *C. histolyticum* counts ↓, *C. lituseburense* counts, *F. prausnitzii* counts ↓, *Bacteroides-Prevotella* counts ↑, IgA-coated *Bacteroides-Prevotella* ↓	**CeD-GFD vs. CeD**: IgG-coated bacteria counts ↓, IgM-coated bacteria counts ↓ **CeD-GFD vs. HC**: IgG-coated bacteria counts ↓, IgM-coated bacteria counts ↓	(De Palma et al. 2010)
CeD-GFD: N=19, median age 9.7 years HC: N=15, median age 10.4 years	FC		**CeD-GFD vs. HC**: *Lactobacillus*↓, *Enterococcus*↓, *Bifidobacteria*↓, *Bacteroides*↑, *Staphylococcus*↑, *Salmonella*↑, *Shigella*↑, *Klebsiella*↑.	(Di Cagno et al. 2011)
CeD: N=20, mean age 4.8 years CeD-GFD: N=20, mean age 5.6 years HC: N=20, mean age 4.5 years	PCR	**(CeD & CeD-GFD) vs. HC**: *Staphylococcus epidemidis* ↑, *S. haemolyticus* ↑ **CeD vs. HC**: *S. aureus* ↓, *Staphylococcus* spp. *diversity* ↑	**CeD-GFD vs. CeD**: *S. aureus* ↑, *S. warneri* ↑, *Staphylococcus* spp. *diversity* ↓ **CeD-GFD vs. HC**: *Staphylococcus warneri* ↓	(Sánchez et al. 2012)

CeD individuals, implying the impaired host defence and barrier function (De Palma et al. 2010).

To date, no consensus on celiac disease-specific microbial signature has been identified due to the inconsistent findings (Table 5 & 6). This might be because of differences in the sample materials (fecal vs. biopsy), variation in the length of gluten-free diet and patients' age, biases in the methodologies (traditional methods vs. high-throughput microarray/sequencing) or sample sizes. It is also not known, whether the observed CeD-associated microbiota changes are primary and play a role in the disease pathogenesis or occur as a consequence of the disease development. The observations that delivery by caesarean section, infantile infections and the use of antibiotics increase the risk of developing CeD (Decker, Hornef, and Stockinger 2011; Mårild et al. 2013; Plot and Amital 2009) indicate that microbiota could play a role in the disease onset. Interestingly, the CeD risk allele HLA-DQ2 was reported to determine the early intestinal microbiota composition and the carriers had lower number of *Bifidobacterium* species and increased levels of several genera belonging to Proteobacteria (Olivares et al. 2014). In order to clarify the role of microbiota in CeD onset, large cohort of subjects with genetic predisposition to CeD should be followed in time from unaffected health status to the development of disease. However, it is a great challenge to accomplish such studies.

2.3.3 Potential roles of microbiota in CeD

The exact mechanisms explaining how intestinal microbiota could affect the development or onset of CeD remain unknown. The suggested pathways involve the modulation of epithelial integrity and innate immunity as well as exacerbation of gliadin-induced immune responses (Verdu, Galipeau, and Jabri 2015).

Gluten contains considerable amounts of proline and glutamine amino acids, which make it resistant to enzymatic digestion in the small intestine (Shan et al. 2002). These long gliadin peptides subsequentially pass through the intestinal mucosa where they are deaminated by tissue transglutaminase 2 (TG2) in LP, captured by DCs which then trigger pro-inflammatory gluten-specific $CD4^+$ T cell responses (Shan et al. 2002; Green and Jabri 2006). These gluten-specific immune responses include the production of IFN-γ, anti-tissue TG (tTG) antibodies, anti-gliadin antibody and anti-endomysial antibodies (EMA) in susceptible individuals (Shan et al. 2002; Green and Jabri 2006). Tissue damage, such as the increased mucosal permeability encountered after the upregulation of zonulin or some other mechanisms triggered during a gastrointestinal infection, might explain why long gliadin peptides could pass through the mucosa to LP (Fasano et al. 2000). The role of intestinal microbiota in the progression, or onset of CeD, could be either positive or negative. *Clostridium* spp., *Streptococcus* spp. and coliforms are the predominant bacteria in the small intestine, and these organsims might contribute to rapid responses to dietary changes (Zoetendal et al. 2012). Furthermore, *Clostridium* spp., particularly members of the *Clostridium* cluster XIVa, not only can transform undigested polysaccharides into butyrate directly, but can also indirectly convert lactate produced by *Streptococcus* spp. to butyrate, which is a crucial metabolite promoting epithelium integrity (Zoetendal et al. 2012). *In vitro*, *Bifidobacterium lactis* and *B. bifidum* have been shown to be protective against gliadin-induced barrier dysfunction in epithelial cell lines (Cinova et al. 2011;

Lindfors et al. 2008). In contrast, *E. coli* and *Shigella* have been demonstrated to increase the translocation of gliadin-peptides into LP (Cinova et al. 2011). Furthermore, *Bacteroides fragilis* and *Akkermansia muciniphila* have been reported to improve epithelial integrity (Reunanen et al. 2015). In addition, commensals as a whole can be considered as a physical barrier against pathogens, while butyrate produced by specific bacteria could strengthen the epithelium barrier function, as discussed already in Section 2.1.2.

Microbiota may affect the progression of CeD via immune modulation, including both the innate and adaptive arms of the immune system (Green and Jabri 2006). The combination of both a enhanced response to bacterial ligands and microbial dysbiosis may lead to cytokine and chemokine profiles that are predominantly pro-inflammatory. For example, duodenal biopsies from Finnish CeD patients were found to have altered duodenal expression of TLRs and their inhibitor which is indicative the involvement of innate immunity and bacterial ligand recognition in CeD (Kalliomäki et al. 2012). Furthermore, carriers of SH2B3, a risk allele for CeD, have enhanced activation of the NOD2 recognition pathway, which is important in bacterial pathogen recognition (Zhernakova et al. 2010). For exploring the exact roles of microbiota in the pathogenesis of CeD, microbiota with and without a pathobiont, *E. coli*, was introduced into germ-free mice with HLA-DQ8-genotype (Galipeau et al. 2015). The presence of *E. coli* exacerbated the development of gluten induced pathology and, if the microbiota was devastated with vancomycin treatment the pathology was further increased in the presence of *E. coli* (Galipeau et al. 2015). The dysbiotic microbiota containing *E.coli* evoked both an increased pro-inflammatory gliadin specific T-cell response and increased IgA anti-gliadin antibody production as compared to microbiota without *E.coli* (Galipeau et al. 2015). Increased IL-15 levels are associated with active CeD (Jabri et al. 2000) and Gram-negative bacteria derived LPS may play a role in stimulating IL-15 production through TLR4 activation (Re et al. 2004) and thus modulating the immunological environment. This might suggest some involvement of LPS-carrying members of the gut microbiota, particularly Proteobacteria, in the IL-15 upregulation. On the other hand, *B. fragilis* might prevent and reverse the intestinal inflammation in CeD by enhancing Treg cells and anti-inflammatory cytokines production. Intrestingly, *B. fragilis* produces polysaccharide A (PSA), which activates TLR2 signalling and it releases PSA-containing outer membrane vesicles to deliver immunoregulatory effects to the host (Shen et al. 2012).

2.4 Methodology to study intestinal microbiota

A detailed characterization of the microbial composition is the first step in the exploration of the role of this complex ecosystem in health and disease. To date, a variety of approaches have been applied. Traditional culture-based methods can detect those bacterial species that can be cultivated on laboratory media, but these kinds of bacteria constitute only 10 to 50% of the microbiota (Eckburg et al. 2005). Great advances have been made through the application of culture-independent techniques, which allow broader and less biased profiling of the microbiota (Zoetendal *et al.*, 2008). The 16S rRNA gene (approximately 1500 bp, 16S rDNA) is the most often used phylogenetic marker (Kuczynski et al. 2012) and the culture-independent methods are generally based on the analysis of this gene. 16S ribosomal RNA is a structural component of the 30S subunit of all prokaryotic

ribosomes. The 16S rRNA gene contains conserved and variable regions. The conserved regions can be used for designing broad spectrum primers and variable regions are valuable for genus/family level classification (Kuczynski et al. 2012). The similarity of the detected 16S rRNA gene sequence to those of cultured bacteria or previously identified sequences represents the basis for analyzing microbiota composition using this approach. Phylotype or OTU (operational taxonomic unit) is used to describe bacterial species detected by 16S rRNA gene sequence analysis, since a true identification of bacteria requires a phenotypic characterization. 16S rRNA gene sequences can be clustered according to their similarities, resulting in different corresponding taxonomic unit (OTU) levels. Cut-off values of 97-99% and 90% similarity between 16S rDNA sequences are often referred to as phylotype or OTU and genus level classification, respectively (Rajilić-Stojanović et al. 2009).

Common 16S-based techniques include fingerprinting techniques, such as denaturing/temperature gradient gel electrophoresis (D/TGGE) and terminal-restriction fragment length polymorphism (T- RFLP) for microbiota community profiling, and quantitative real-time PCR (qPCR) and fluorescent *in situ* hybridization (FISH) for targeted detection and quantification of specific bacterial taxa or species (Satokari et al. 2003). The low-resolution microbiota community profiling techniques, such as D/TGGE and T-RFLP have been replaced by high throughput (HT) approaches. On the other hand, qPCR is highly sensitive and requires a relatively small amount of template, and thus it has been extensively used for verifying the results obtained from HT microbiota analysis (Bacchetti De Gregoris et al. 2011).

DNA microarrays and next generation sequencing (NGS) are commonly used HT methods in RNA/DNA analysis (Kuczynski et al. 2012). Human Intestinal Tract Chip (HITChip), a bacterial phylogenetic microarray, has been applied extensively in human intestinal microbiota studies investigating both healthy subjects and individuals affected by various diseases, such as atopy, obesity, IBS and IBD (Korpela et al. 2016; Jalanka et al. 2015; Nylund et al. 2013; Rajilić-Stojanović et al. 2009). NGS techniques have been developed to allow massive parallel amplification of a vast number of (partial) 16S/18S rRNA genes or total community DNA from complex microbial ecosystems and facilitate rapid and holistic characterization. When the whole intestinal microbiota DNA is determined by NGS, this approach is termed metagenomics (or whole-genome shotgun sequencing) (Lepage et al. 2013; Nielsen and Ji 2015). The main difference of metagenomics from the 16S rRNA-based approach is the detection of the whole genetic potential i.e. collective genomes and capacity (functions) of the sampled ecosystem instead of only its phylogenetic composition. Thus, metagenomics generates both phylogenetic and genomic information, and thus it also provides insights into the potential functions. Miseq and Hiseq (Illumina), Ion Torrent (Life Technologies) and Single-molecule real-time sequencing (SMRT, Pacific Biosciences) are currently used NGS platforms. The details of these platforms have been recently reviewed in (Reuter, Spacek, and Snyder 2015; Lepage et al. 2013; Nielsen and Ji 2015). The essential steps in 16S rRNA gene based microbiota analysis and specific challenges in each step are summarized in Table 7. Notably, as reviewed in Lozupone et al. (2013), the 16S rRNA region, sample preparation protocol and sequencing platforms are all major resources for technical variation, which can affect the results obtained. Therefore, experimental design considerations, such as the selection of control populations, adequate cohort sizes and standardized protocols, including both wet-

and dry lab procedures, are recommended for controlling for unwanted confounding effects.

Table 7. Steps and challenges in 16S rRNA gene based microbiota analysis

Step		Key point	Reference
Experimental design	**Study population**	**Confounding effects:** age and antibiotics usage are important confounders in microbiota research. For example, intestinal microbiota is considered to be relatively stable only after 3 years of age. Therefore, the age between the compared populations should not differ too extensively when young children are being studied. Regarding the confounding effect from antibiotics, it might require one year or even longer recovery time, for gut microbiota to return to baseline composition, or it may never return. Therefore, any antibiotic intake should be carefully recorded and taken into account in the data analysis. Other confounders include e.g. diet and genetic background.	(Yatsunenko et al. 2012; Macfarlane 2014; Dethlefsen and Relman 2011)
	16S approach	**16S rRNA gene copy number variation**: Varied copy numbers of 16S rRNA genes can be present in the genomes of different strains and species, and more than one type of 16S rRNA gene sequence has also been reported to exist in some strains. Thus, 16S rRNA gene quantitation is only indicative of cell numbers	(Sun et al. 2013; Větrovský and Baldrian 2013)
		Targeted regions: sequencing V1-V3 region has been found to generate higher richness than V3-V5, but less diversity within *Bifidoacteriaceae*.	(Group 2012; Huse et al. 2012)
Sample collection	**Sample type**	**Choose representative bacterial community**: Microbiota differ in different body compartments.	Table 1 and (Kuczynski et al. 2012)
		Homogeneity of stool sample: Microbiota composition, especially the low abundance taxa, can vary between the outside and inside of the stool i.e. between the liquid and particle-form of the same feces. Therefore, it is important to homogenize the specimen.	(Walker et al. 2008; Swidsinski et al. 2010)
	Storage	**Short-/long-term**: The key is to avoid freeze-thawing cycles before DNA extraction, since these are detrimental for DNA integrity**.** Freezing may favor the extraction of DNA from Gram-positive bacteria. Long-term storage should be at -80˚C, but there is no golden standard for short-term storage	(Thomas, Clark, and Doré 2015)
Wet lab	**DNA extraction**	**Cell lysis**: Repeated bead beating is recommended to efficiently extract DNA from both Gram-negative and Gram-positive bacteria	(Salonen et al. 2010; Yu and Morrison 2004)
		Extraction kits: both manual extraction	(Kennedy et

		methods and commercial kits have been used. Also (semi)-automated extraction devices have been used. The extraction method can influence the results.	al. 2014)
	Libary preparation	**Primers for amplifying 16S rRNA gene**: The match between so-called universal primers and bacterial target DNA can vary according to bacterial species. Therefore, it is recommended to use adequately degenerated primers.	(Group 2012; Kuczynski et al. 2012)
	Microarray and sequencing	**Cross-hybridization and sequencing errors**: The cross-hybridization observed between incorrect combination between probes and non-target molecules, and chimeras formed during PCR amplification are the critical errors in microarray and sequencing, respectively. Corresponding bioinformatics tools are available for correcting these errors.	(Schloss, Gevers, and Westcott 2011; Koltai and Weingarten-Baror 2008)
Dry lab	**Pre-processing**	**Quality control**: Reference samples and/or spiked genes are always needed	(Haas et al. 2011; Reuter, Spacek, and Snyder 2015)
		Sequence identification: There is a difference in using reference-based or *de novo* approach in the taxonomic assignment of sequence information. In a reference-based approach, a high quality database is required, such as SILVA and Greengenes.	(Kuczynski et al. 2012)
		Sequencing depth: The number of reads obtained (library size) can vary extensively between samples, which can even be greater than any biological differences in some studies. Therefore, the normalization of the library size or controlling for its effect is necessary when comparing different samples	(Gihring, Green, and Schadt 2012)
	Statistical analysis	**Complex data and multiple comparisons**: high throughput data are high dimensional, sparse and contain fewer samples than the number of variables. Therefore, on one hand, it is difficult to explore inter- and intra-individual variation. On the other hand, multiple comparisons introduce false positive p-values.	(Tsilimigras and Fodor 2016; Kuczynski et al. 2012)
Wet lab	**qPCR validation**	**Extracting health/disease-specific bacterial signature**: 16S approaches are not accurate at the species/strain level. Therefore, high throughput analysis can be considered as a discovery tool and results usually need validation by qPCR especially for low abundance species/strain- level quantification.	(Bacchetti De Gregoris et al. 2011)

	Characterization of health/disease-specific bacterial isolates	**Translation between experimental models**: The functional exploration of specific bacterial strains starts from *in vitro* studies, followed by controlled model systems, such as *in vitro* cell lines and *ex vivo* models and finally *in vivo* models/trials. The results from controlled systems and *in vivo* trials quite often differ from each other and therefore, model systems need to be constantly improved.	(Bermudez-Brito et al. 2013)

Although high-throughput methods, especially metagenomics are useful tools for characterizing genetic potential and compositional changes of the gut microbiota, this method can only deliver the potential functions of gut microbiota. Therefore, other omics-approaches are often applied to reveal in greater detail the activities of the microbial community at different functional levels. For example, meta-transcriptomics/proteomics/bolomics, are holistic approaches which focus on gene expression, protein and metabolites of the microbiota respectively, as reviewed in Kolmeder and de Vos (2014). Once health/disease specific microbes, such as potential probiotics, have been discovered by HT approaches, candidate-bacteria should be verified by qPCR or isolated by culturing.Furthermore, since the properties of bacteria, including probiotics, are species/strain specific, the assessment of strain properties is mandatory in different controlled experimental model systems before proceeding to *in vivo* human interventions (Marteau 2011). In such studies, various *in vitro* cell lines, such as Caco 2 and HT 29 for epithelial interaction studies, *ex vivo* model systems and *in vivo* animal trials have been exploited (Bermudez-Brito et al. 2013; Tsilingiri and Rescigno 2012; Doron and Snydman 2015).

Similarly, both targeted and holistic approaches have been applied in identifying health/disease specific host gene expression signatures in both clinical samples and experimental models. The reverse transcription polymerase chain reaction (RT-PCR), is a fast and straightforward method for targeted (focused) analysis of specific genes, such as immune mediator molecules, or PRRs (Korpelainen et al. 2014). Nowadays, it is still extensively applied, and even the expression level of specific gene can be identified, but selecting the specific genes to analyze the activation of different pathways is challenging. Therefore, omics-approaches such as RNA sequencing exhibit superiority in providing a holistic view and enabling also pathway analysis approaches (Korpelainen et al. 2014). A further challenge in analysing and interpreting host omics-data can be traced to the fact that clinical samples such as mucosal biopsies commonly contain mixed cell types. For example, both immune cells and enterocytes with different functions, as discussed previously in Section 2.1.2, may express the same PRRs.

3. AIM OF THE STUDY

- To compare the intestinal microbiota in healthy young children of 1–4 years of age to that as healthy adults (study I)
- To characterize the development and stability of intestinal microbiota in healthy pre-school age children (study II)
- To identify mucosa-associated microbial signatures and to explore the role of host-microbiota interactions in healthy and CeD children (study III)

4. MATERIALS AND METHODS

4.1 Study subjects and samples

The studies were approved by the University of North Carolina (UNC) Internal Review Board (I and II) (Fig. 9) and the ethical committee of the Hospital District of Southwest Finland (III). All adult subjects and guardians of the children provided consent. Notably, children with treated Celiac disease could not be included as a control group in study III, because in Finland, it is not permissible to perform an invasive sampling during esophagogastro-duodenoscopy in order to take follow-up biopsies from children, if their celiac serology has turned negative within two years after the GFD treatment. A summary table of study subjects in the cross-sectional studies I and III is provided in Table 8.

The children in studies I and II represent a subset of subjects from a larger synbiotic intervention trial (ClinicalTrials.gov: NCT00653705) (Ringel-Kulka et al. 2015). The baseline and follow-up samples of these children were used in studies I and II respectively. Study I is a cross-sectional study for the comparison of intestinal microbiota composition between children and adults from the same geographical region in US (Fig. 9). Study II focuses on the characterization of fecal microbiota development in children (Fig. 9).

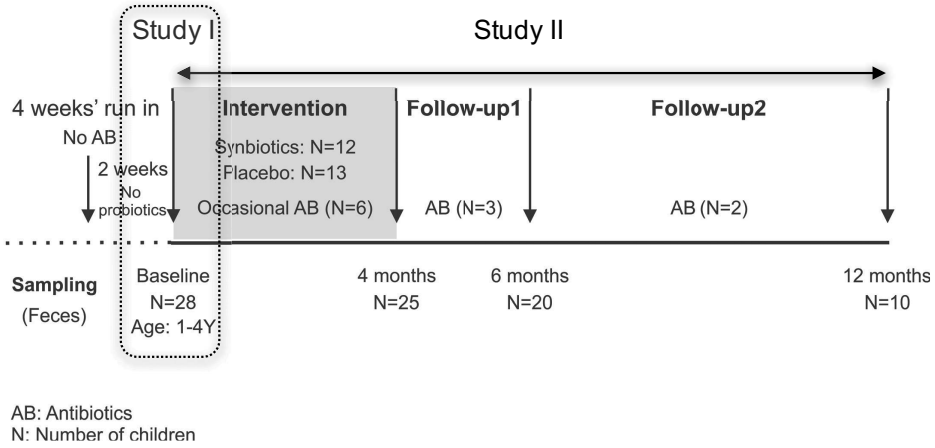

Figure 9. Design of the synbiotic intervention trial and samples used in studies I & II (modified from Fig. S1 in publication II). After sampling at baseline, the children were enrolled in a four-months synbiotic intervention trial. In synbiotic intervention group, the children took the product containing probiotic strain *Bifidobacterium animalis* subsp. *lactis* (BB-12), yogurt starter strains *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, together with prebiotic inulin. While in placebo intervention group, the children drank acidified milk. The details are described in (Ringel-Kulka et al. 2015). Follow-up fecal samples were collected for 1 year, i.e. immediately after the intervention, and 2 and 8 months after the cessation of the intervention (Fig. 9). Both children and adults had no history of antibiotic treatment in the previous 4 weeks before the intervention, and no history of probiotics consumption during the 2 weeks prior to the start of the intervention.

Table 8. Characteristics of study subjects in the cross-sectional studies I and III

Study	Sample type	Subjects with different health status	Subjects in different age categories	Age (median±SD)	Gender (F/M)	Race	BMI category*	BF vs. FF
I	Feces 1 sample/subject	Healthy (N=51)	Children (N=28)	2.7±0.9 (1-4Y)	15/13	Black: N=7; Hispanic: N=3; Pacific Islander: N=1; White: N=16; NR: N=1	Normal: N=14; Overweight: N=8; Obese: N=6	BF: N=23; FF: N=4; NR: N=1
			Adults (N=23)	28±13.2 (21-60Y)	19/4	Asian: N=1; Black: N=2; White: N=20	Normal: N=17; Overweight: N=1; Obese: N=5	NR
III	Duodenal biopsy 1 sample/subject	Healthy (HC, N=10)	Children (N=10)	8.5±3.8 (4-16Y)	6/4	White: all Finnish	NR	NR
		Treated celiac disease (TCeD, N=6)	Adults (N=6)	46±11.4 (30-60Y)	3/3	White: all Finnish	NR	NR
		Active celiac disease (CeD, N=10)	Children (N=10)	9.5±4.1 (3-14Y)	6/4	White: all Finnish	NR	NR

Study I: *Body mass index (BMI) was calculated per standard definition (kg/m^2). The weight and height of adults and children was measured when they visited the study clinic and at daycare center respectively. Adult BMI was categorized as healthy (18.5-24.9), overweight (25-29.9) or obese (≥30). Children's BMI percentile was calculated and adjusted by age and sex according to the World Health Organization (WHO) standard and then categorized as healthy (5-84.9%), overweight (85%-94.9%) or obese (≥ 95%) (Ringel-Kulka et al. 2013).

Study III: HC subjects underwent esophagogastroduodenoscopy due to gastrointestinal complaints or other reasons. TCeD had been on a GFD for at least 1 year. The HC and TCeD subjects were determined by negative celiac serology and Marsh 0 lesions characterized by normal small intestinal mucosa. In active CeD patients, positive celiac serology markers, such as anti-tissue transglutaminase antibodies and/or anti-endomysial antibodies; and Marsh III lesions characterized by villus atrophy and crypt hyperplasia were detected in duodenal biopsy.

4.2 Analytical methods

Both fecal DNA (I & II) and duodenal tissue RNA/DNA (III) were studied. The methods used in the analysis are detailed in Tables 8-10. Briefly, the sample preparation, storage and RNA/DNA extraction followed previously described protocols (Carroll et al. 2011; Kalliomäki et al. 2012). Subsequently, the bacterial phylogenetic microarray HITChip was applied to produce microbiota profiles (I, II and III) (Rajilić-Stojanović et al. 2009) with slightly modified protocols to deal with the specific types of samples (Table 9 & 10). Reverse transcriptase quantitative PCR (RT-qPCR) was used to study the host mucosal gene expression (III) (Table 10 & 11) as described previously (Kalliomäki et al. 2012). Data analysis were conducted using parametric or non-parametric statistics for clinical data, intestinal microbiota and host gene expression analysis (I, II and III) (Table 11). The bioinformatic and statistical analysis were performed with R statistical software packages "stats", "vegan", "multcomp" and "nlme". In addition, qPCR was used for quantitative analysis of specific bacterial taxa in study I (Table 9).

HITChip is specifically designed for characterizing human intestinal microbiota diversity and composition (Rajilić-Stojanović et al. 2009). It utilizes approximately 4,800 oligonucleotide probes targeting on 1038 bacterial phylotypes (<98% identity). Each phylotype is classified by multiple probes targeting the V1 and V6 hyper-variable regions of the 16S rRNA gene. These probes are categorized at three levels of specificity: level 1 (L1, phylum/order like sequence taxa), level 2 (L2, genus-like taxa, >90% similarity), and level 3 (L3, >98% similarity). The relative change of the hybridization signal of phylotype-specific probes has been shown to be directly proportional to the quantitative change of the respective phylotype in a complex ecosystem (Rajilic-Stojanovic et al., 2009).

Table 9. Methods used in the procedures of microbiota analysis in the thesis.

Steps	Method	Study	Reference
Sample preparation			
DNA extraction	**Fecal DNA**: Bead-beating integrated with phenol-chloroform for DNA extraction and Qiagen DNeasy blood and tissue extraction kit for DNA purification.	I, II	(Carroll et al. 2011)
	Tissue DNA: Bead-beating integrated with Qiagen DNeasy blood and tissue extraction kit.	III	(Kalliomäki et al. 2012)
DNA quality control	NanoDrop ND-1000 spectrophotometer.	I, II, III	(Carroll et al. 2011; Kalliomäki et al. 2012)
HITChip microbiota profiling			
Amplification of 16S rRNA gene	**Fecal DNA**: T7 prom-Bact-27- FOR and Uni-1492-REV primers, targeting on V1 to V9 regions in 16S rRNA gene	I, II	(Rajilić-Stojanović et al. 2009)

	Tissue DNA: T7 prom-Bact-27- FOR and Uni-1369-REV primers, targeting on V1 to V8 regions in 16S rRNA gene	III	(Iwamoto et al. 2000)
***In vitro* transcription, dye labelling, fragmentation, hybridization**	PCR products were transcribed *in vitro* to RNA and labeled with dye Cy3 and Cy5, followed by fragmentation and hybridization. At least two samples from the same subject, each carrying one dye at a time, were hybridized to two different arrays until they passed the quality control and produced reproducible microarray profile.	I, II, III	(Rajilić-Stojanović et al. 2009)
Extraction of intensity value	Qualified by Agilent Feature Extraction software (version 10.7.3.1).	I, II, III	(Rajilić-Stojanović et al. 2009)
Pre-processing			
Quality filtering	Pearson correlation between technical replicates was determined by using an in house R script and coefficient 0.95 was used as the cut-off value for reproducibility.	I, II	(Lahti et al. 2014)
	Pearson correlation coefficient 0.94 was used as the cut-off value for technical replicates of microarray profiles from duodenal biopsies.	III	(Lahti et al. 2014)
Between-sample normalization in HITChip data	Min-max algorithm.	I, II, III	(Han 2011; Bolstad et al. 2003)
Background signal subtraction in HITChip data	The biological signal intensity was defined as 25% higher than the average value of signal intensity of 500 probes with the lowest signals.	III	(Rajilić-Stojanović et al. 2009)
Phylogenetic characterization of microbial ecosystem			
Diversity	Estimated by Simpson's reciprocal index and Shannon indices on log10-transformed probe level data.	I, II, III	(Oksanen et al. 2015)
Richness	Quantified by the number of responding oligos.	II	(Satokari et al. 2014)
Evenness	Estimated by Pielou's evenness index on log10-transformed probe level data.	II	(McCune and Grace 2002)
Stability	Estimated by Pearson's correlation coefficient between consecutive sampling points on log10-transformed probe level data.	II	(Satokari et al. 2014)
Composition	Estimated by summing the signal intensities for probes targeting a L1/L2 taxa.	I, II, III	(Jalanka-Tuovinen et al. 2011)

Core microbiota at L2	Core L2 HITChip bacterial taxa were defined as shared by at least 70% of the subjects with at least 0.1% relative abundance.	II	Study II
Functional prediction of microbiota contents			
Butyrate-producing bacteria	Estimated by summarizing the relative abundances of 10 HITChip L2 taxa, which have been reported to be butyrate-producing bacteria.	II	Study II
MAMP prediction	Estimated by summing up the bacterial abundance of G+, G-, or flagellated or high GC% L2 HITChip taxa. The relative abundance of these bacteria was used to reflect their corresponding MAMP ligand load.	III	Study III
Validation of HITChip microarray result			
***Bifidobacterium* spp.**	Performed in SYBR Green integrated qPCR, and quantified by normalizing to standard DNA.	I	(Bartosch et al. 2004; Carroll et al. 2011)

G^+: Gram-positive bacteria; $Gram^-$: Gram-negative bacteria; high-GC% bacteria: genomic GC% content is more than 58%.

Table 10. Methods used in host gene expression analysis.

Steps	Method	Study	Reference
RNA stabilization	RNAlater	III	(Kalliomäki et al. 2012)
RNA extraction	Qiagen RNeasy Plus Mini-kit	III	
RNA quality control	Bio-Rad Expression system	III	
qRT-PCR	Taqman gene expression assay	III	
Quantification of gene expression data	Relative changes as compared to endogenous control (18S RNA)	III	

Table 11. Statistical methods used in data analysis.

Steps	Method	Study	Reference
Clustering/classification	Hierarchical clustering with complete linkage algorithm	I	(Legendre and Legendre 1998)

	Cross validation + Random forest	III	(Breiman Leo 2001)
Dimensionality reduction	Principal component analysis	I, III	(Venables and Ripley 2003)
	Redundancy analysis	I, III	(Legendre and Legendre 1998)
Data normality test	Shapiro-Wilk normality test	I, II, III	(Royston 1995)
Test of significance	**Parametric statistics**: t-test, ANOVA, linear (mixed) model	I, II, III	(Chambers and Hastie 1991; Pinheiro, Bates, and R-core 2015)
	Non-parametric statistics: Wilcoxon rank-sum test, Kruskal-Wallis test	II, III	(Siegel and Jr 1988)
	Permutation test on the constraints after redundancy analysis	I, III	(Legendre and Legendre 1998)
	P-value correction for multiple testing: Tukey's Honest Significant Differences post-hoc analysis, Benjamini-Hochberg false discovery rate correction	I, II, III	(Yandell 1997; Benjamini et al. 2001)
Correlation testing	Spearman or Pearson correlation coefficient	I, II, III	(Chambers and Hastie 1991; Siegel and Jr 1988)

5. RESULTS AND DISCUSSION

5.1 Age is the major determinant of fecal microbiota development of young children (Study I and II)

The studies (I and II) examined the relationship between the effects of many variables, some of which might be confounding factors that should be controlled before one can draw any general conclusions in future observational clinical studies. As illustrated in Fig.9, the samples used in studies I and II were a subset from a 2-arm, randomized, double-blind and placebo-controlled trial but sampling biases were still present in gender, race, BMI category and BF or FF (Table 8) (Ringel-Kulka et al. 2015). Therefore, it was important to evaluate the potential confounding effects of these variables on microbiota in both the baseline and subsequent follow-up samples.

We identified no significant effects of gender, race and BMI category on the structure of microbial community in either of our study cohorts (children or adults), as demonstrated by visual examination in PCA and no statistical difference by using permutation test on RDA analysed constraints (Fig. S2-S4/I, as referred to the

Supplementary Figure 2-4 in publication I). Furthermore, no significant confounder effect was found at the bacterial genus-like level after applying linear regression models. Although in adults the abundance of several bacterial taxa was associated with gender, race and BMI category, the abundance of these particular taxa was not significantly different between children and adults (Table 1/I). In addition, no correlation was found between the history or duration of BF and the abundances of the bacterial genus-like level taxa in children, including bifidobacteria (study I). These results suggested that the confounding effects of the above mentioned variables were minimal in the data.

In the longitudinal study II, the effects of the synbiotic supplements, occasional antibiotic courses, zBMI and age were estimated by linear mixed effect models. Except for age, the other factors had no or only a minor effect on the overall microbiota profiles except for several specific phylum-level bacterial taxa (Table S5/II).

The synbiotic supplement was found to exert a minor effect on fecal microbiota in the intervention group i.e. it only increased the stability of one phylum-like taxa i.e. Actinobacteria, out of a total of the 22 phylum-like taxa present in the samples. The limited response in the overall structure of fecal microbiota is in agreement with the previous observations in several probiotic intervention studies conducted in both children and adults (Gerritsen et al. 2011; Kim et al. 2013; Lahti et al. 2013). The increased stability of Actinobacteria is likely due to inulin, as it is known that inulin supports the growth of bifidobacteria which is the main taxa within the Actinobacteria phylum in the intestine (Salazar et al. 2015; Veereman 2007).

The relative abundance of *Clostridium* cluster XIVa was found to be reduced by antibiotics in the first sample taken after the course (LME P=0.02, Fig. S2/II). In addition, the stability of the *Clostridium* cluster I was reduced by the use of antibiotics. These results are consistent with previous studies where antibiotics was found to reduce microbiota stability and diversity (Rehman et al. 2012; Panda et al. 2014). However, the abundance of *Clostridium* cluster XIVa was not significantly lower in the subsequent follow-up samples as compared to the baseline samples. This indicates that after the cessation of antibiotic therapy in study II, which are penicillin-type antibiotics, the *Clostridium* cluster XIVa had partially recovered from a lower level of abundance after the antibiotic course (Fig. S2b/II). Unlike the long term effect of macrolides, which have wider spectrum than penicillin, the penicillin-type antibiotics including amoxicillin have been reported to exert a weaker effect on both overall microbial richness and specific microbiota abundance (Korpela et al. 2016). Furthermore, even with repeated administrations of penicillin, no significant reduction in richness was found at six months after the treatment (Korpela et al. 2016). Therefore, a single antibiotic course using amoxicillin produced only temporary effect as a partial recovery eventually occurred within weeks or months. The effects of synbiotic consumption and antibiotics were considered as covariates when estimating the temporal development of microbiota.

5.2 Comparison of fecal microbiota composition in healthy Western young children and adults (Study I)

5.2.1 Similarities of microbiota composition between children and adults

When comparing the fecal microbiota composition in healthy young US children (denoted as group C) and adults (denoted as group A), similarities for certain bacterial taxa were found at both phylum-like and genus-like levels. Firmicutes (C:80.67%, A:81.12%), Bacteroidetes (C:5%, A:13%) and Actinobacteria (C:12.5%, A:3.4%) were observed to be the most predominant phyla in both age groups. Previously, similar findings have been reported i.e. that Firmicutes and Bacteroidetes are the major bacterial taxa of intestinal microbiota in both children and adults (Yatsunenko et al. 2012; Eckburg et al. 2005).

Phylogenetically, Firmicutes contains a broad range of genera; these are often divided into phylogenetic sub-groups, which have also distinctive functional characteristics. For example, *Clostridium* clusters IV and XIVa contain butyrate-producers (Duncan et al. 2002). *Clostridium* cluster XIVa was found to be the most predominant taxa in both the children and adult groups, with almost equal abundances (50.4% vs. 54.9% respectively; $p = 0.37$), suggesting that this phylum may have already been stabilized at an early age i.e. before 4 years of age. The early establishment of the *Clostridium* cluster XIVa was further supported by the observation that 18 out of 19 *Clostridium* cluster XIVa genus-like taxa did not differ significantly in their relative abundance between children and adults. *Ruminococcus obeum* et rel. was noted as the most predominant genus-like taxa in both adults and children, with the same level of relative abundance (11.6% in A and 13.9% in C; $p>0.05$). The trend of *Clostridium* cluster XIVa was further explored in more detail in study II, by dividing the children into different age categories to assess their microbiota development; these results will be discussed later in Section 5.3.1.

5.2.2 Dissimilarities of microbial composition between children and adults

At the genus-like level, the structure of the fecal microbiota community in healthy US children aged 1-4 years old was significantly different from that of adults from the same geographic region as revealed by both unsupervised PCA and supervised RDA ($p = 0.003$) analyses. The community level differences between children and adults were further highlighted by significant differences in relative abundances of 26 genus-like bacterial taxa, which all had a relative abundance of at least 0.01% of the total bacterial population and thus, can be considered as major taxa within the ecosystem. Notably, significantly higher levels of bifidobacteria were detected in children by both microarray analysis (C:11% versus A:3%, $p = 0.00008$) and qPCR (2.7-fold higher abundance, $p< 0.006$). In contrast to some previous reports that the children in the US may lack bifidobacteria (Palmer et al. 2007), our study showed that higher levels of bifidobacteria were present also in the US children, similar to the situation in European and Japanese children reported previously (Adlerberth and Wold 2009).

Five phylum-like bacterial taxa displayed significant differences between children and adults. For example, children in general had a higher abundance of Bacilli, which contains three predominant genera, *Streptococcus bovis*, *S. mitis* and *S. intermedius* et rel. (Table 1/I). On the other hand, *Clostridium* cluster IV had a higher relative abundance in adults than in young children (24% vs. 16% respectively; $p = 0.02$). Furthermore, all dominant genus-like level taxa within the *Clostridium* cluster IV consistently exhibited either equal or higher abundance in adults. However, all the bacterial taxa within this phylum-like level taxa showed very high variability in terms of the relative abundance between the individuals of the same age, with *F. prausnitzii* et rel being the most variable taxa in young children (data not shown). These findings indicate that the microbial compositional transformation within *Clostridium* cluster IV has not yet reached its ultimate level in young children. Similarly, Bacteroidetes showed a significantly higher abundance in adults than in children (A: 13% vs. C: 4.7%, $p = 0.02$). Adults had a range of 2.4–6.3-fold higher abundance of all dominant genus-like level taxa within this phylum than children. Similar to *F. prausnitzii* et rel, all of the taxa in Bacteroidetes displayed high variance in terms of abundance in both children and adults. Actinobacteria were observed to be the third most abundant phylum-like level taxa in children, while being significantly less abundant than in adults (A: 3.4% vs C: 12.5%, $p<0.01$). More detailed development patterns of these major phyla were characterized in study II in an attempt to gain a better understanding of their early establishment.

5.3 Characterization of fecal microbiota development in 1-5-year old children (Study II)

5.3.1 Temporal stability and resilience

Intestinal microbiota is less diverse up to 5 years of age than in adults

No change in microbiota diversity was observed in children from approximately 1 to 5 years of age. Moreover, the microbiota diversity measured at the HITChip oligo level was significantly lower in all age categories (1-2Y, 2-3Y, 3-4Y, 4-5Y) of children as compared to adults (Fig. 5/I and Fig. 1/II, $p<0.01$). It was shown that microbiota diversity, including richness and evenness, underwent fluctuations when the children were 1–5 years of age, and it had not yet reach its zenith by the age of 5 years (Fig. 1/II). These results contrast the recent findings that microbial diversity of children in United States has progressed to a similar level than in adults by the age of three years (Yatsunenko et al. 2012; Schloss et al. 2014). Furthermore, these US children harbour a less diverse microbiota in comparison to their counterparts from developing countries in South America and Africa (Yatsunenko et al. 2012). We hypothesized that the Western lifestyle with its excessively hygienic conditions, diet and the use of antibiotics may influence the diversification of intestinal microbiota in infants and young children. Recently, this hypothesis was tested in mice fed with either a Western diet (high in fat and simple carbohydrates, low in fibre) or a more traditional diet containing a high proportion of fibre (Sonnenburg et al. 2016). The consumption of the Western diet was found to result in a less diverse microbiota, especially the lost taxa in *Bacteroidale*s, which is the fibre-utilizing order. The loss of diversity was further

shown to be restored within a single generation if the animals were provided with the traditional diet, but it became irreversible after several generations with the same diet i.e. there was a permanent loss of microbial taxa that are passed on from one generation to the next (Sonnenburg et al. 2016). Therefore, two scenarios can be considered for Western children; they may either develop a more diverse microbiota later in life or their microbiota might unlikely reach the same level of diversity to that of earlier generations due to the permanent loss of certain microbial taxa, such as *Bacteroidale*s, which has taken place over several generations.

Major phyla undergo gradual transformation from 1 to 5 years of age

Although no change was found in bacterial diversity in children from 1 to 5 years of age (Fig. 1/II), a gradual transformation of microbiota was observed as the stability of the overall bacterial community increased with age ($p=0.03$ in LME, Fig.2/II). Further, discordant phyla development, with different microbial abundance and stability from children towards adults was detected, as shown in Table 12.

Although the stability of overall microbiota profile increased from 1 to 5 years of age (Fig. 2/II), it was still remarkably lower than that previously observed for Western adults as investigated with the same microarray technique (Jalanka-Tuovinen et al. 2011; Rajilić-Stojanović et al. 2012; Fuentes et al. 2014; Satokari et al. 2014). Furthermore, we found heterogeneity in the stability of different phylum-like level taxa (Fig. 4/II, Table 12). For example, *Clostridium* clusters XIVa has temporally unstable profiles, although it is the most abundant phylum-level taxa and has already been established at 1-2Y. In contrast, although *Clostridium* clusters IV has temporally unstable profiles as well, its abundance is still increasing from 1 to 5 years of age. The increase of stability of these bacterial taxa was not obvious, suggesting that Firmicutes might be in the process of rearrangement in children and that the phylum is expected to reach a mature status only at a later age. On the contrary, the other major phyla including Actinobacteria, Bacteroidetes and Proteobacteria had higher stability than *Clostridium* clusters in children. Interestingly, we found that Bacteroidetes, more specifically *Bacteroides* spp. was the only phylum that invariably showed increasing stability from 1 to 5 years of age (Fig. 5c/II, $p=0.04$), suggesting that Bacteroidetes might be the main driver of microbiota stability toward an established individual-specific microbiota assembly from infancy to adulthood. Our results are in line with a previous study conducted on a Swedish cohort, where the process of microbiota maturation was also observed to proceed beyond 1-2 years of age (Bäckhed et al. 2015). On the other hand, we found that *Clostridium* cluster XIVa and Proteobacteria had reached an adult-like abundance level already by 1–2 years of age (Table 12). This observation is more consistent with the conventional view that intestinal microbiota usually becomes adult-like within the first 3 years of life (Yatsunenko et al. 2012). Thus far, most studies have been cross-sectional focusing on the abundance of bacterial taxa. Our longitudinal data demonstrates that stability should be also assessed to gain an evolutionary understanding. The establishment of *Clostridium* cluster XIVa at high abundance at an early age highlights the importance of these bacterial taxa in the symbiotic relationship between the human host and the intestinal microbiota. On the other hand, it might indicate that diet already in toddler age provides stable amount of nutrients utilized by *Clostridium* cluster XIVa. Overall, we concluded that major

intestinal phyla are in the process of developing into an adult-type composition at an individualized pace, and the overall duration of microbiota maturation seems to take up more than 5 years.

Table 12. Summary of the temporal development of different phylum-level bacterial taxa from 1 to 5 years of age (modified from Table 1 in publication II)

Phylum-level taxa	Relative abundance at 1-2y vs. adults	Change in relative abundance 1-5y	Stability* at 1-5y	Overall status
Actinobacteria	Higher	No change	High, no change	Child-like, stable
Bacilli	Higher	No change	High, no change	Child-like, stable
Bacteroidetes	No difference	No change	Moderate, increase	Developing
***Clostridium* cluster I**	Higher	No change	Moderate, no change	Child-like, relatively stable
***Clostridium* cluster III**	Lower	Increase	High, no change	Developing
***Clostridium* cluster IV**	Lower	Increase tendency	Low, no change	Child-like, unstable
***Clostridium* cluster XI**	No difference	Increase	Moderate, increase tendency	Developing
***Clostridium* cluster XIVa**	No difference	No change	Low, no change	Adult-like, unstable
Proteobacteria	No difference	No change	Moderate, no change	Adult-like, relatively stable

Abbreviations: y, years
*Stability was assessed as Pearson correlation of oligoprofiles between consecutive time points

Previously, higher levels of bifidobacteria have been found in infants in comparison to adults (Roger et al. 2010; Arrieta et al. 2014; Bergström et al. 2014). In our study, we showed that bifidobacteria had a higher abundance in children aged 1-2 years than in adults ($p<0.01$, study I and II) and moreover, that this kind of child-like abundance level did not change significantly from 2 to 5 years. As discussed in Section 5.1.2, synbiotic intervention exerts limited effect in the study cohort and it could not explain the elevated levels of bifidobacteria. In a recent Danish study, *Bifidobacterium* spp. was decreased from 9 months to 3 years of age, which might due to the cessation of breastfeeding at 9 to 18 months (Bergström et al. 2014). On the other hand, another study using a microarray technique to assess microbiota in adolescents reported that the adolescent group showed higher (nearly two-fold) levels of *Bifidobacterium* spp. compared to the adult group (9% vs, 5.4% respectively; $p<0.01$), pointing to decrease of bifidobacteria from adolescents to adult (Agans et al. 2011). The intermediate level of relative abundance in adolescents as compared to that in young children (11%) and adults (3% and 5.4%) may suggest a gradual decrease in *Bifidobacterium* spp. levels starting from infancy to adulthood. This decrease in *Bifidobacterium* spp. abundance might persistent after adulthood to death, since lower abundance of *Bifidobacterium* spp. was observed in elderly than adults (Claesson et al. 2011). The findings by Agans et al (Agans et al. 2011) are in line with our observation that in childhood, the intestinal microbiota is not yet assembled in an adult-type manner. *Bifidobacterium* spp. are

generally considered as important candidates for probiotic strains, as several studies have suggested that they may exert health-promoting functions e.g. by strengthening epithelial integrity and having anti-inflammatory properties (Jungersen et al. 2014; Hsieh et al. 2015). Furthermore, bifidobacteria can cross-feed butyrate producing bacteria by acting as primary degraders, which partially hydrolyze polysaccharides which then act as the secondary substrates for the butyrate-producing bacteria (De Vuyst and Leroy 2011).

Analysis of children categorized into different age groups revealed differences also in the levels of inter-individual similarity. We found that the inter-individual similarity peaked at 3-4 years of age in our study cohort, while lowest in adults (Fig. S7/II), suggesting a more similar microbiota within this age group. The result may reflect the longer consumptions of standardized meals and longer exposure to the overall similar microbial environment in a daycare center of the older children as compared to their younger counterparts. This concept is supported by the observation that diet and environment may affect the gut microbiota shortly after institutionalization such as in daycare or healthcare centers (Claesson et al. 2012).

5.3.2 Emergence of core microbiota

The core microbiota has been investigated in adults in order to reveal major bacterial taxa in subjects beyond their individuality (Sekelja et al. 2011; Huse et al. 2012; Turnbaugh et al. 2009). The development and establishment of the core microbiota starting from infancy have remained largely unknown. In study II, the core microbiota of children was investigated and compared to that of adults. We found that a common core, comprising of 18-25 genus-like taxa belonging to Actinobacteria, Bacilli, *Clostridium* clusters IV and XIVa constituted more than 70% of the total microbiota with a stable level of abundance in all age categories of children and adults (Fig. 10; Fig. S8/II). We further determined the timeline of the emergence of the core microbiota and found that the assembly of the core microbiota may have started already at 1 year of age. The expansion of the core microbiota from infancy to adulthood was observed with the increased number of genus-level taxa, from 18 in 1-2-year-old children to adults aged 25 respectively. This dynamic development was also reported in a recent study, although it described a much larger core consisting of 49 genera in 1-year old infants (Vallès et al. 2014). The smaller size of core microbiota in our study might be due to the more stringent filtering process of the genus-like taxa, i.e. we applied a threshold of >0.1% of relative abundance and >70% prevalence to filter out the unrepresentative taxa. The composition of the core microbiota was not static, as we found that Bifidobacterium, *Streptoccus mitis* et rel. and *Streptococcus bovis* et rel. in the children core were finally taken up by *Oscillospira guillermondii* et rel., *Butyrivibrio crossotus* et rel. and *Clostridium stercorarium* et rel. in adults (Fig.10). Our results provide evidence for the start of the early establishment of a core microbiota, which holds the possibility of compositional changes in the assembly of core microbiota due to environmental factors and physiological changes after childhood.

Notably, we found that butyrate-producing bacteria increased steadily in their relative abundance to the adult-like level before 5 years of age (p=0.02, study II). A recent nutritional study reported >30% increase of absolute intakes of protein, fibre during the first two years of life (Lioret et al. 2013). Therefore, the increasing level of butyrate-producing bacteria might due to increasing fibre intake. Furthermore, bacterial taxa producing butyrate were also increased in the children's core microbiota (Fig. 10). This was interpreted as evidence of the ongoing diversification of this essential functional taxa in healthy children. Butyrate-producing bacteria provide butyrate as the major energy source for enterocytes and thus they support the mucosal physiology by stimulating motility, mucous secretion, sodium and water absorption, as well as regulating epithelial barrier and immunity functions of the IECs (Leonel and Alvarez-Leite 2012). Recently, Jalanka et al. (2016) reported a therapeutic microbiota core that could explain the treatment efficacy of fecal microbiota transplantation for recurrent *Clostridium difficile* infection in adults. Interestingly, the bacterial taxa included in this therapeutic core have 64% overlap with the healthy adult core microbiota in study II. Importantly, six out of eight butyrate producers that were included in the core in study II were also present in the therapeutic core defined by Jalanka et al. (2016). This concurrence indicates that core microbiota and specific taxa therein can be considered as keystone members for maintaining intestinal homeostasis and host health.

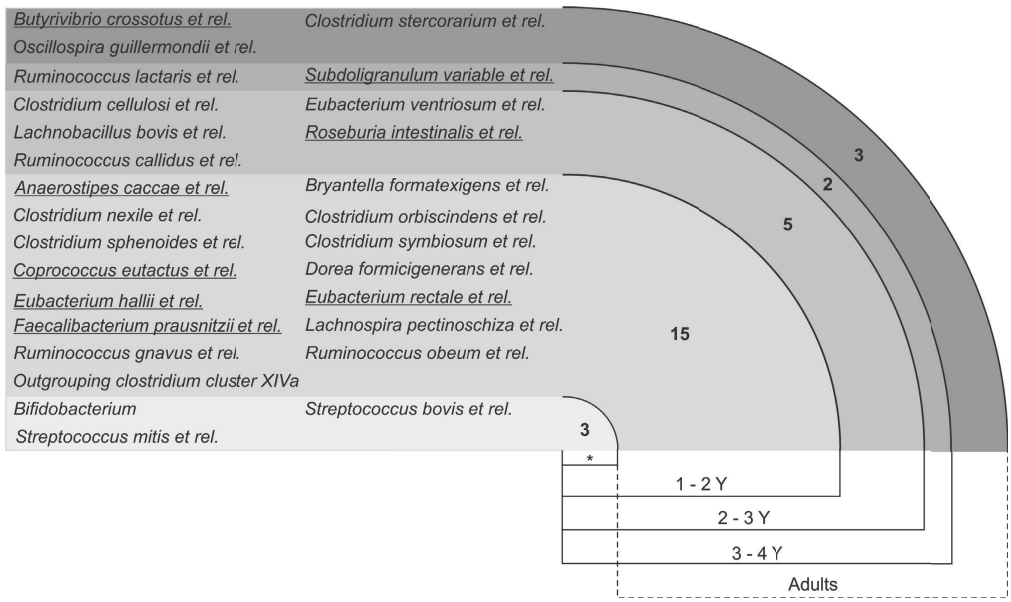

Figure 10. The development of core microbiota (adapted from Fig. 6 in publication II).

5.4 Duodenal mucosal homeostasis in celiac disease and healthy children (Study III)

5.4.1 Microbiota profiles of duodenal mucosa

In study III, we investigated the microbiota profiles in duodenal mucosa in HC and CeD children as well as explored host-microbe interactions by examining the mucosal gene expression and. Altogether 13 phylum-like and 65 genus-like taxa were detected in healthy duodenal mucosal biopsies (Table 1/III), which is substantially fewer than the number previously observed in fecal samples in a pediatric population analyzed with the same profiling platform i.e. 22 and 130 phylum- and genus-like level taxa respectively (study I and II). The major phylum-like level taxa in duodenal biopsies were Proteobacteria, Bacilli, Bacteroidetes and *Clostridium* cluster XIVa (Fig. 2/III). The predominant genus-like level taxa were *Sutterella wadsworthensis* et rel., *Streptococcus mitis* et rel., *Aquabacterium*, *Streptococcus bovis* et rel., *Streptococcus intermedius* et rel., and *Prevotella melaninogenica* et rel. Notably, the genus Streptococcus accounted for about 30% of the total duodenal microbiota in HC subjects, suggesting a major contribution of streptococci in the small intestinal microbiota, which has been observed previously in ileostomy samples in adults (Zoetendal et al. 2012; Sullivan et al. 2003). Furthermore, *S. wadsworthensis* et rel. was detected as the most abundant genus-like taxa within Proteobacteria, in line with previous findings in human mucosal samples in both pediatric and adult populations (Mukhopadhya et al. 2011; Hansen et al. 2013). It has been suggested that *Sutterella* spp. might play a role in the pathogenesis of IBD, autism, Down syndrome and metabolic syndrome (Williams et al. 2011; Biagi et al. 2014; Lim et al. 2016; Hansen et al. 2013), while other studies do not provide supporting evidence for this hypothesis (Mukhopadhya et al. 2011; Son et al. 2015). In a recent study, the interaction of three *Sutterella* species with intestinal epithelium was examined *in vitro,* and the results suggested *Sutterella* spp. to be mutualistic i.e. it was thought to maintain the immune system in an alert-state, like other commensals, but not to evoke a strong inflammatory reaction (Hippala et al. 2016).

5.4.2 Health- and celiac disease associated microbial signatures

The phylum- and genus-like bacterial compositions, and total bacterial diversity in duodenal mucosa were similar between the HC and CeD groups (Table 13). Furthermore, the microbial community structures were not separated between HC and CeD subjects, as revealed by using PCA and RDA analyses (Table 12, Fig. 11, and Fig. S1-2/III). In this study, no significant difference in single bacterial abundance is consistent with previous observations, which have compared duodenal microbiota in HC and CeD subjects (Ou et al. 2009; Kalliomäki et al. 2012). However, in contrast to our results, microbial dysbiosis with increased level of facultative anaerobes to obligate anaerobes, and/or elevated microbial diversity and total bacterial counts (Nadal et a. 2007; Schippa et al. 2010) have been reported in the duodenal mucosa in active CeD children in comparison to their HC counterparts. These altered bacterial taxa in CeD children are higher levels of *Staphylococcus* and Proteobacteria, particularly *E.coli* (Nadal et al. 2007; Collado et al. 2009; Sánchez et al. 2013) and increased *Bacteroides*

with decreased bifidobacteria (Nadal et al. 2007; Collado et al. 2009). Thus, some evidence exists both in support and against the possibility of microbiota dysbiosis in CeD. In an attempt to understand the collective interactions between bacterial taxa, a random forest prediction model was implemented to extract several genus-like taxa contributing to the separation of HC and CeD (Table 13, Table 1/III). For example, *P. melaninogenica* et rel., *Haemophilus* spp. and *Serratia* spp. had higher relative abundances in CeD compared to HC, while the other five bacterial taxa were more abundant in HC samples (Table 13). The functional relevance of these bacterial taxa in the differentiation of CeD from HC remains unknown and may be worthy of investigation in follow-up studies. It is still unclear, whether the possible microbiota changes detected in CeD are primary and contribute to the pathology of the disease or alternatively, the changes may occur as a consequence of the disease. Thus far, longitudinal microbiota data has been unable to reveal a possible link between microbiota composition including specific members within the microbiota and the disease development.

It has to be taken into account that CeD profoundly affects the morphology, physiology and immunology of the small intestinal epithelium, and this represents a completely different ecological niche for bacteria as compared to a normal healthy mucosa. Furthermore, the possibility that microbes are involved in the etiology is also supported by the association found between infections and the risk of developing CeD (Plot and Amital 2009; Verdu et al. 2007). In this respect, both bacterial and viral infections have been suspected, such as *Campylobacter jejuni* (Verdu et al. 2007), Adenovirus 12 and Hepatitis C virus (Plot and Amital 2009). Therefore, further studies focusing on explaoring functional contents of all microbes, including virus are warranted to clarify the role of microbiota in CeD.

Table 13. Summary of key findings in the duodenal samples in study III.

	CeD vs. HC	**TCeD vs. HC**
Microbiota	No difference in overall microbiota community No difference in the abundance of single bacterial taxa No difference in bacterial diversity No difference in the estimated microbial ligand (MAMP) content for TLRs <u>A sub-group of bacteria separate CeD and HC:</u> **HC**: *Prevotella oralis* et rel. ↑, *Proteus* et rel. ↑, *Clostridium stercorarium* et rel. ↑, *Ruminococcus bromii* et rel. ↑, *Papillibacter cinnamivorans* et rel. ↑ **CeD**: *Prevotella melaninogenia* et rel. ↑, *Haemophilus*↑, *Serratia*↑	No data
Expression of tight junction proteins and immune-associated genes	**HC**: positive correlation between ZO-1 and TLR2, IL-10/IFN-γ ↑, TLR2↑, Tollip↑ **CeD**: CXCR6 ↑, IL-10↑, IFN-γ↑, IL-8↑, TLR9↑	**TCeD**: CXCR6↑, IFN-γ↑ **HC**: IL-10/IFN-γ ↑, TLR2↑

5.4.3 Dys-regulated host-microbiota interaction

Microbes can be stratified by their ligand specificity (so-called MAMP content, see Section 2.1.2), which is recognized by host immune cells or IECs, i.e. they play a role especially in the innate immune response. Similar to our result on microbiota profiles, no significant differences were found in the general MAMP content between HC and CeD subjects, suggesting that there is an overall similarity in the bacterial ligand loads.

It has been reported that TLR signaling can affect epithelial barrier function through the expression of TJ proteins, gap junction proteins, MUC2 proteins and antimicrobial peptides such as RegIIIγ (Verdu et al. 2007; Ey et al. 2009). Specifically, *in vitro* stimulation of IECs by TLR2 has been characterized by an increased level of Cx43 synthesis (a marker for gap junction proteins), an apical re-organization of ZO-1 (a marker for TJ proteins) and trans-epithelial resistance, which reflects the strength of tight junctions between IECs and barrier function (Cario, Gerken, and Podolsky 2004; Cario, Gerken, and Podolsky 2007; Ey et al. 2009)

As previously reported for the same subjects, the expression of TLR2 was significantly reduced in both CeD and TCeD subjects when they were compared to HC (Kalliomäki et al. 2012). In addition, the downregulation of Tollip protein, the regulator of TLRs was observed in CeD as compared to HC (Kalliomäki et al. 2012). The expressions of TLR9 and IL-8, a marker of intestinal inflammation, were also found to be increased in CeD (Kalliomäki et al. 2012). TLR2 is expressed in both immune cells and IECs; this could be demonstrated via the LTA content of specific microbes (Abreu 2010). We found similar LTA contents, as assessed by the proportion of Gram-positive bacteria in the microbiota, between HC and CeD (Table 13, Table 1/III). Therefore, the net signaling through TLR2 might be increased in HC. Furthermore, the ZO-1 expression correlated positively with the level of TLR2 expression in the HC group ($p = 0.02$, $rho = 0.88$, Table 13, Fig. 5/III), supporting an earlier finding, which demonstrated that the epithelial barrier was more permeable in CeD subjects as compared to HC due to the decreased expression of tight junction proteins (Pizzuti et al. 2004). In our study, although no differences in the levels of expressions of proteins ZO-1 and Cx43 were detected between CeD and HC (Table A2/III), significantly lower ZO-1 expression was found in CeD in comparison to TCeD (Fig. 5/III). The only correlation that was found between the abundance of bacterial taxa and expression of the studied host genes was the negative correlation between *S. moorei* et rel. and the expression of Cx43 protein. However, no difference of *S. moorei* et rel. was found in the abundance between CeD and HC. Also the expression of Cx43 did not differ between CeD and HC. Thus, *S. moorei* does not seem to be associated specifically with CeD, but the result implies that *S. moorei* may affect intestinal barrier, which could be studied further by using *in vitro* models.

As discussed in Section 2.3.3, alterations of both microbiota and the expression of host receptors recognizaing bacteria might involve in the pathogenesis of celiac disease, even though no conclusive microbial signatures have been reported thus far. CXCL16 is an important scavenger receptor in APCs for mediating phagocytosis of bacteria and it is a critical chemokine in leukocyte trafficking when there is inflammation (Darash-Yahana et al. 2009; Shimaoka et al. 2003; Uza et al. 2011). Specifically, the co-expression of CXCL16 and its receptor CXCR6 in natural killer T cells and Th1-

polarized CD4 T cells has been implicated in IBD-associated inflammation (Uza et al. 2011). In our study, the expression level of the cytokine, CXCL16, was found to be similar in CeD, TCeD and HC, whereas the expression of its receptor CXCR6 was higher in CeD and TCeD as compared to HC. CXCL16 has dual functions being a transmembrane adhesion molecule and a soluble chemokine (Abel et al. 2004). Both the membrane-bound forms of CXCL16 and its receptor CXCR6 have been found to be expressed not only in dendritic cells and macrophages and T cells respectively, but also in IECs (Abel et al. 2004; Hase et al. 2006; Diegelmann et al. 2010). Previously, increased intestinal CXCL16 expression has been observed in the colonic biopsies of Crohn´s disease patients due to immune cell infiltration (Diegelmann et al. 2010). In study III, an increased expression of CXCR6 was observed not only in the inflamed mucosa of CeD but also in TCeD without immune cell infiltration, suggesting that the increase level of CXCR6 originated from epithelial cells. However, the contribution of mucosa associated immune cells to the increased expression of CXCR6 could not be excluded as whole biopsies consist of many different cell types. The CXCL16-CXCR6 chemokine-receptor systems has been shown to activate several distinctive signaling pathways in IEC, contributing to the integrity of epithelium through the regulation of mucosal innate and adaptive immune systems (Diegelmann et al. 2010). These are the first results demonstrating an increased duodenal expression of CXCR6 in CeD subjects, evidence of the important role of CXCL16-CXCR6 for mucosal immunity also in small intestine of CeD subjects.

The high GC content of a bacterial genome correlates with a higher number of potentially immunostimulatory CpG motifs in the genome, which are ligands for TLR9 in immune cells and IEC (Kant et al. 2014). Therefore, the GC content reflects the load of TLR9 ligands in the microbiota. The average genomic GC content (GC%) of the total microbiota, as well as the proportion of high-GC% bacteria was found to be comparable between the HC and CeD groups (Table 13, Table 1/III). Consequently, HC and CeD subjects are likely to harbor similar loads of TLR9 ligands. Therefore, the higher expression of TLR9 found in the previous study using the same cohort in CeD might reflect an increased net signaling through TLR9. In immune cells, TLR9 stimulation is known to trigger Th1-proned immune responses (Krieg 2002; Yu et al. 2007). Furthermore, an *in vitro* study demonstrated that apical TLR9 stimulation of IECs led to an increased expression of IFN-γ and IL-10, but not TNF-α from peripheral blood mononuclear cells (PBMCs) on the basolateral side of the cell membrane (de Kivit et al. 2011). In our CeD patients, increased mucosal expressions of IL-10 and IFN-γ were also observed (Fig. 7/III). IL-10 and IFN-γ have been associated with the activity of gliadin-reactive T-cells after gluten/gliadin stimulation both *in vivo* and *in vitro* (Forsberg et al. 2002; Salvati et al. 2005; Gianfrani et al. 2006; Nilsen et al. 1995; Nilsen et al. 1998). We found that the expression levels of IFN-γ in the duodenal mucosa were increased also in TCeD subjects adhering to a gluten-free diet as compared to HC (Table 13, Fig. 7/III), indicating that there must be an additional gluten/gliadin-independent route of stimulation. Similarly to previous studies (de Kivit et al. 2011; Nilsen et al. 1995; Nilsen et al. 1998), TNF-α expression was found to be unaffected in CeD and TCeD as compared to HC. The ratio of IL-10 to IFN-γ transcripts was clearly reduced in both CeD and TCeD patients as compared to HC (Table 13, Fig. 7/III), suggesting that although IL-10 expression is increased there is still a significant inclination towards a Th1 response in both untreated and treated CeD

patients. In conclusion, based on our results, I hypothesize that TLR9 signalling in the small intestine may contribute to the persistent activation of Th1 signalling pathway in CeD, as indicated by the increased levels of IFN-γ found in the CeD children even though they were consuming a gluten-free diet (Lahdenperä et al. 2011). The mechanisms of TLR9 signalling pathway might be mediated via the direct stimulation of immune cells and/or IECs.

Optimally, the same group of patients, before and after GFD (TCeD) or a control group of pediatric TCeD patients would have been included in the study. In study III, a control group of pediatric TCeD could not be included, because in Finland follow-up biopsies are not taken from children after the implementation of GFD, if the treatment has been effective. CeD children and TCeD adults are likely to have differences in their microbiota composition also due to the age (Nistal et al. 2012; Cucchiara et al. 2009), and microbiota can influence the physiological status of the host including epithelial gene expression (Maynard et al. 2012; Wells et al. 2011). However, we found that the expression of a number of mucosa-associated genes, TLR2, TLR9 (Kalliomäki et al. 2012), CXCR6, IL-10 and IFN-γ (study III) were increased in both CeD children and TCeD adults as compared to HC children. Thus, the altered expression of these genes may be intrinsic to CeD and independent of the age and microbiota composition.

The strengths of this study are the comprehensive microbiota analysis by high throughput bacterial phylogenetic microarray, the use duodenal biopsies (and not feces) to study microbiota changes in CeD, which primarily affects the small intestinal mucosa and the gene expression analysis from the same individuals, which allows the assessment of correlations between microbiota and expression of relevant mucosa-associated genes. The weaknesses of this study are the small study groups and the inability to include TCeD children as a comparison group for ethical reasons explained above. Thus far, duodenal mucosa-associated microbiota in pediatric CeD has been studied from biopsies of 168 active CeD, 103 TCeD and 90 HC children (Nadal et al. 2007; Collado et al. 2009; Ou et al. 2009; Schippa et al. 2010; c; Di Cagno et al. 2011; Kalliomäki et al. 2012; Nistal et al. 2012; Sánchez et al. 2013), but only three studies have utilized a high throughput technique (pyrosequencing) to comprehensively assess the duodenal mucosal microbiota (Nistal et al. 2012; Sánchez et al. 2013). Although the study groups in the present work are small, our results from high-throughput microbiota profiling, like the results by Nistal et al. (2012) and Sánchez et al. (2013), give a more in depth view of the duodenal microbiota regarding the bacterial groups inhabiting duodenal mucosa and the overall diversity and underline the need to for further studies in order to improve the current understanding on possible links between microbiota and pediatric CeD.

6. CONCLUSION AND FUTURE PERSPECTIVES

In studies I and II, as compared to adults a less diverse microbiota was found in children of 1-5 years of age. Actinobacteria, Bacilli and *Clostridium* cluster IV retain the abundance characterized as child-like, while some other taxa already started to progression to adult-type assembly. The overall microbiota stability increased with age, which seems to be driven by the increasing stability of Bacteroidetes. In children, a

common core of microbiota was identified, consisting of 18 to 25 abundant genus-level taxa, such as butyrate-producing bacteria. The microbiota core was shown to be developing toward an adult-type composition. In conclusion, we found that the intestinal microbiota in children is still developing, suggesting that the establishment adult-like profile may proceed even after the age of 5 years. The discordant development patterns observed with different taxa might reflect physiological development pattern in children (Table. 14).

Table 14. Microbiota characteristics in healthy children at 1-5 years of age

Microbiota features	Characteristics
Diversity	Lower than in adults
Stability	Lower than in adults, but increasing
Composition	Difference bacterial taxa "mature" at different age
Core microbiota	Smaller core than in adults, but expanding

Our results in study III suggest that intestinal microbiota and the host-microbe cross-talk play a role in the CeD. No significant difference between CeD and HC was demonstrated in the overall microbiota composition as evaluated by its phylogeny or the potential immunomodulation capacity of microbiota as assessed by the estimated MAMP content. However, eight genus-like bacterial taxa, which separate CeD and HC may play a role in the disease by affecting the epithelial integrity. The involvement of impaired barrier function in CeD is further supported by the elevated expression of TLR2 and its positive correlation with tight junction protein in healthy individuals. Elevated expression levels of IL10, IFN-γ and CXCR6 were found in both treated and active CeD groups, suggesting that the Th1 responsiveness in CeD subjects may be attributable to the increased level of TLR9 signaling, even with adherence to a GFD.

All our findings in healthy and CeD pediatric populations should be further investigated in larger cohorts in a more controlled environment before any conclusions can be generalized. Functional genomics approaches, such as metagenomics, transcriptomics and proteomics will become ever more important for investigating mechanisms involved in host-microbe interactions. These will require a more comprehensive understanding of the molecular mechanisms behind microbial symbiosis or dysbiosis for their human host. The specific microbial signatures of both symbiosis and dysbiosis could provide potential functional diagnostic and therapeutic biomarkers. These markers could then be applied for promoting the healthy/natural microbiota development, as well as for improving health in specific diseases via enteric microbiota modulation. For this purpose, bacteriotherapies, either involving probiotics (single bacterial species), cocktails (combination of bacterial species) or fecal microbiota transplantation (whole bacteria community) will be future cutting-edge research topics. There are experiments underway evaluating the potential beneficial role of *Bifidobacterium* spp. in various diseases ranging from neuropsychiatric disorders to obesity (Kalliomäki et al. 2008; Pärtty et al. 2015), as well as in CeD by combatting protecting gliadin-induced barrier dysfunctions (Cinova et al. 2011; Lindfors et al. 2008). Long-term studies that integrate microbial features, dietary data and pathological biomarkers from healthy status to pathological alterations in a large

cohort of subjects with genetic predisposition will be critical for clarifying how microbial effects are intertwined with the onset of disease.

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